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For and on behalf of RWS Group Ltd

The 16th day of February 2005

54 Muteins of the bilin-binding protein

57 The invention relates to muteins of the bilin-binding protein which are capable of binding digoxigenin and to fusion proteins of such muteins, to methods for preparing muteins of this kind and their fusion proteins and also to the use thereof for detecting or binding biomolecules labeled with digoxigenin. The invention especially relates to a polypeptide, selected from muteins of the bilin-binding protein, which is characterized in that it (a) is able to bind digoxigenin or digoxigenin conjugates, (b) does not bind ouabain, testosterone and 4-aminofluorescein and (c) has an amino acid substitution at at least one of the sequence positions 28, 31, 34, 35, 36, 37, 58, 60, 69, 88, 90, 95, 97, 114, 116, 125 and 127 of the bilin-binding protein. Due to their simple molecular structure, the muteins of the invention have advantages for production and use in comparison with antibodies against the digoxigenin group.

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Description

The present invention relates to muteins of the bilin-binding protein which are capable of binding digoxigenin and to fusion proteins of such muteins, to methods for preparing muteins of this kind and their fusion proteins and also to the use thereof for detecting or binding biomolecules labeled with digoxigenin.

In molecular biology, the digoxigenin group is these days a very common instrument for nonradioactive detection of nucleic acids, proteins and other biomolecules. For this purpose, the biomolecule is, mostly covalently, modified with a reactive digoxigenin derivative, thus allowing subsequent detection of the molecule using an antibody directed against the digoxigenin group or a conjugate of an appropriate antibody fragment and a reporter enzyme, according to generally used methods in biochemistry.

The skilled worker knows quite a number of reactive digoxigenin derivatives which are partially also commercially available. For example, digoxigenin-3-O-methylcarbonyl-ε-aminocaproic acid N-hydroxysuccinimide ester (DIG-NHS), digoxigenin-3-O-succinyl-ε-amino-caproic acid N-hydroxysuccinimide ester and 3-amino-3-deoxydigoxigenin-hemisuccinimide succinimidyl ester are suitable for covalent coupling to proteins, in particular to the amino groups of exposed lysine side chains. Using 3-iodoacetylamino-3-deoxydigoxigenin it is possible to label especially thiol groups in proteins or in other biomolecules selectively with the digoxigenin group. It is possible to couple synthetic oligodeoxynucleotides to the same reactive digoxigenin derivatives, as long as they have been provided with suitable free amino or thiol groups during synthesis.

In addition, cis-platinum complexes of digoxigenin derivatives (DIG Chem-Link reagent) or digoxigenin derivatives containing carbodiimide groups (disclosed in the European patent specification EP 0 806 431 A2) are suitable for direct labeling of nucleic acids. Alternatively, it is possible in the case of deoxyribonucleic acids to label said deoxyribonucleic acids during a matrix-dependent enzymic synthesis with the aid of a DNA polymerase and a deoxynucleotide triphosphate coupled to the digoxigenin group, for example digoxigenin-11-dUTP, digoxigenin-11-ddUTP or digoxigenin-16-dATP. Analogously, digoxigenin-11-UTP is suitable for incorporation into enzymically synthesized RNA. Moreover, it is possible to label oligodeoxynucleotides with the digoxigenin group directly in the automated DNA synthesis by using suitable activated building blocks, for example "virtual nucleotides". Digoxigenin group-coupled nucleic acids of this kind are suitable as nonradioactive gene probes for detection of complementary nucleotide sequences by hybridization, for example in Northern or Southern blots (disclosed in the European patent specification EP 0 324 474 A1).

Digoxigenin group-labeled proteins or glycoproteins are particularly useful for determining, for example, relevant antigens or antibodies directed thereagainst in immunochemical assay methods such as ELISA (enzyme-linked immunosorbent assay). The biomolecule conjugated with the digoxigenin group is actually detected using an anti-digoxigenin antibody, normally in the form of a conjugate of the Fab fragment of said antibody with a suitable enzyme, such as, for example, alkaline phosphatase or horseradish peroxidase, as label. The enzymic activity then serves for quantification via catalysis of a chromogenic, fluorogenic or chemiluminescent reaction. Various antibodies against the digoxigenin group are known (Mudgett-Hunter et al.,

J. Immunol. 129 (1982), 1165-1172; Jeffrey et al., J. Mol. Biol. 248 (1995), 344-360).

The use of antibodies, however, has several disadvantages. Thus, preparation of monoclonal antibodies in hybridoma cell cultures is complicated, and proteolysis to give the Fab fragment and also production of conjugates with reporter enzymes requires additional difficult process steps. But even the production of antibodies by genetic engineering is not simple, and the main reason for this is that antibodies as well as antigen-binding fragments thereof are composed of two different polypeptide chains in a structurally complicated manner. For genetic manipulation of antibodies it is therefore necessary to handle two genes simultaneously. Moreover, the yield of correctly folded antibody fragments produced by genetic engineering is often low. As is known to the skilled worker, this is even more so when recombinant fusion proteins are to be prepared from Fab fragments of antibodies and enzymes.

It was therefore the object of the invention to develop alternative polypeptide reagents for detection of the digoxigenin group, which can be produced in a simple manner.

In an evolutive research approach, it has surprisingly been found now that muteins of the bilin-binding protein which is structurally based on a single polypeptide chain (Schmidt and Skerra, Eur. J. Biochem. 219 (1994), 855-863) are suitable for detecting the digoxigenin group by high-affinity binding, with digoxigenin recognition being astoundingly selective compared with other steroids.

The present invention thus relates to a polypeptide, selected from muteins of the bilin-binding protein, which is characterized in that it

(a) is able to bind digoxigenin or digoxigenin conjugates,

(b) does not bind ouabain, testosterone and 4-aminofluorescein and

(c) has an amino acid substitution at at least one of the sequence positions 28, 31, 34, 35, 36, 37, 58, 60, 69, 88, 90, 95, 97, 114, 116, 125 and 127 of the bilin-binding protein.

Outside the region of amino acid positions 28, 31, 34, 35, 36, 37, 58, 60, 69, 88, 90, 95, 97, 114, 116, 125 and 127 the muteins of the present invention may correspond to the amino acid sequence of the bilin-binding protein from *Pieris brassicae*. On the other hand, the amino acid sequence of the polypeptides of the invention may have differences to the bilin-binding protein also besides said positions. Bilin-binding protein sequence variants of this kind comprise naturally occurring and also artificially generated variants, and the deviations mean substitutions, insertions, deletions of amino acid residues and also N- and/or C-terminal additions.

For example, the inventive muteins of the bilin-binding protein may have amino acid substitutions which prevent oligomerization of the bilin-binding protein, such as the Asn(1)->Asp substitution, or suppress proteolytic cleavage within the polypeptide chain, which may occur during production in *E. coli*, such as, for example, the Lys(87)->Ser substitution. Furthermore, it is possible to introduce the mutations Asn(21)->Gln and Lys(135)->Met into the nucleic acids coding for the muteins of the bilin-binding protein, in order to facilitate, for example, cloning of a gene segment via two new *Bst*XI restriction cleavage sites at these positions. Likewise, the present invention relates to the specific introduction of amino acid substitutions within or outside the said positions, in order to generally improve particular properties of the mutein

of the invention, for example its folding stability or folding efficiency or its resistance to proteases.

The ability of the polypeptides of the invention to bind digoxigenin or digoxigenin conjugates can be determined by common methods, for example ELISA, fluorescence titration, titration calorimetry, surface plasmon resonance measurements or blotting methods, for example Western blotting, Southern blotting or Northern blotting. Blotting methods may be used in order to transfer conjugates of digoxigenin with proteins or nucleic acids to a membrane and then detect said conjugates using one of the muteins of the invention, a conjugate of this mutein or a fusion protein of this mutein.

A quantitative parameter for binding affinity is provided by established thermodynamic parameters such as, for example, the affinity constant or dissociation constant for the complex of mutein and bound ligand, for example digoxigenin. However, it is also possible to determine the binding ability qualitatively, for example based on the intensity of a binding signal due to a chromogenic reaction or of a colored precipitate which is obtained with the aid of one of said blotting methods.

Preferred muteins of the invention are obtainable in a two-stage evolutive process. Random mutagenesis of the bilin-binding protein and repeated selection of muteins with digoxigenin group affinity from this library, using free digoxigenin for competitive concentration, provides muteins of the bilin-binding protein which recognize the digoxigenin group, but the affinity is still comparatively low. Renewed mutagenesis of such a mutein at amino acid positions 28, 31, 34, 35, 36 and 37, now followed by a repeated concentration by formation of a complex with the digoxigenin group and by subsequent dissociation of the complex formed in

acidic medium, then results in obtaining muteins having substantially higher affinity for the digoxigenin group.

Surprisingly, it has now been found that the affinity constant between such polypeptides of the invention and digoxigenin is at least 10^7 M^{-1}. This means in other words that the dissociation constant of the complex of the polypeptide of the invention and digoxigenin is 100 nM or less. Individual examples even show dissociation constants of 35 nM or less, as illustrated in the examples.

Besides digoxigenin, the inventive muteins of the bilin-binding protein can also bind digoxigenin derivatives as ligands, for example digoxin, digitoxin or digitoxigenin. Furthermore, the inventive muteins of the bilin-binding protein may bind conjugates of said chemical compounds, i.e. nucleic acids, polypeptides, carbohydrates, other natural or synthetic biomolecules, macromolecules or low molecular weight compounds which are covalently linked or linked via a metal complex to digoxigenin, digoxin, digitoxin or digitoxigenin. Preference is given to using for the preparation of such conjugates the reactive derivatives of digoxigenin, digoxin, digitoxin or digitoxigenin, which are known to the skilled worker and are stated, for example, further above.

Preferred muteins of the invention, which were obtained by the two-stage process described, show, compared with the affinity for digoxigenin, an even higher affinity for digitoxin or digitoxigenin whose steroid system differs from that of digoxigenin only by the absence of a hydroxyl group. Surprisingly, these muteins show distinctive specificity with respect to the digoxigenin or digitoxigenin group, and this is shown by the fact that other steroids or steroid groups such as ouabain or testosterone are bound with much less affinity, if

at all. Fluorescein derivatives such as 4-amino-fluorescein, too, are evidently not bound. This means that ouabain, testosterone or 4-aminofluorescein in each case have a dissociation constant of at least 10 µM, preferably at least 100 µM for the inventive muteins of the bilin-binding protein.

This property of specificity distinguishes said muteins considerably from other muteins of the bilin-binding protein and also from antibodies directed against the digoxigenin group, such as, for example, antibody 26-10 (Chen et al., Protein Eng. 12 (1999), 349-356) which binds ouabain with substantial affinity, and gives the inventive muteins of the bilin-binding protein a particular advantage. It is surprising that particularly the additional amino acid substitutions at positions 28, 31, 34, 35, 36 and 37 lead to the preferred muteins of the bilin-binding protein. Preference is therefore given to those muteins which carry at least one or all of the amino acid substitutions Glu(28)->Gln, Lys(31)->Ala, Asn(34)->Asp, Ser(35)->His, Val(36)->Ile and Glu(37)->Thr.

Particularly preferred muteins of the invention carry, when compared to the biling-binding protein, at least one of the amino acid substitutions selected from Glu(28)->Gln, Lys(31)->Ala, Asn(34)->Asp, Ser(35)->His, Val(36)->Ile, Glu(37)->Thr, Asn(58)->Arg, His(60)->Ser, Ile(69)->Ser, Leu(88)->Tyr, Tyr(90)->Ile, Lys(95)->Gln, Asn(97)->Gly, Tyr(114)->Phe, Lys(116)->Ser, Gln(125)->Met and Phe(127)->Leu. The representation chosen indicates in each case first the amino acid in the natural bilin-binding protein (SWISS-PROT database accession code P09464) together with the sequence position for the mature polypeptide in brackets, and the corresponding amino acid in a mutein of the invention is stated after the arrow. Very particularly preferred muteins according to this invention carry all of the amino acid substitutions mentioned.

Surprisingly, bilin-binding protein position 93 is unchanged in the muteins of the invention, although this amino acid, too, had been affected by the mutagenesis for preparing the random library. Preferred muteins of the bilin-binding protein therefore carry the amino acid Val at said position.

It is an advantage for particular detection methods to use the muteins of the bilin-binding protein of the present invention in a labeled form. Accordingly, this invention further relates to a polypeptide of the invention, which is characterized in that it carries at least one label. Suitable labeling groups are known to the skilled worker and include enzymic label, radioacctive label, fluorescent label, chromophoric label, (bio)luminescent label or a label containing haptens, biotin, metal complexes, metals or colloidal gold. Very generally, labeling is possible with substances or enzymes which generate a determinable substance in a chemical or enzymatic reaction. In this connection it is possible to couple all known labels for antibodies to the muteins of the invention, too.

A possibility which is particularly advantageous for practical application is to use the inventive muteins of the bilin-binding protein in the form of fusion proteins. Techniques for preparing such fusion proteins by means of genetic engineering methods are known to the skilled worker. Suitable fusion partners for the muteins of the invention would be enzymes and other polypeptides, proteins or protein domains. Such fusions would be suitable for providing the mutein of the bilin-binding protein with additional properties such as, for example, enzymic activity or affinity for other molecules such as proteins, macromolecules or low molecular weight ligands.

Examples of possible fusions are those with enzymes which catalyze chromogenic or fluorogenic reactions or

may be used for the release of cytotoxic agents. Further examples for fusion partners which may be advantageous in practice are binding domains such as the albumin-binding domain or the immunoglobulin-binding domain of protein G or protein A, antibody fragments, oligomerization domains, toxins or other binding proteins and functional parts thereof and also affinity peptides such as, for example, Strep tag or Strep tag II (Schmidt et al., J. Mol. Biol. 255 (1996), 753-766). Suitable fusion partners are also proteins having particular chromogenic or fluorogenic properties, such as, for example, green fluorescent protein. Another suitable fusion partner would be coat protein III of a filamentous bacteriophage such as M13, f1 or fd, or a fragment of said coat protein.

Very generally, the term fusion protein is intended here to mean also those inventive muteins of the bilin-binding protein, which are equipped with a signal sequence. Signal sequences on the N terminus of the polypeptide of the invention may serve the purpose of directing said polypeptide during biosynthesis into a particular cell compartment, for example the *E. coli* periplasm or the lumen of the endoplasmic reticulum of a eukaryotic cell, or into the medium surrounding the cell. The signal sequence is typically cleaved off in the process by a signal peptidase. In addition, it is also possible to use other signal or targeting sequences which need not necessarily be located on the N terminus of the polypeptide and which make it possible to locate said polypeptide in specific cell compartments. A preferred signal sequence for secretion into the *E. coli* periplasm is the ompA signal sequence. A large number of further signal sequences and also targeting sequences are known in the prior art.

An advantage of the inventive muteins of the bilin-binding protein is the suitability of both their N terminus and their C terminus for preparing fusion

proteins. In contrast to antibodies in which the N terminus of both the light and the heavy immunoglobulin chain is in spatial proximity to the antigen binding site, it is possible to use in the polypeptides of the invention both ends of the polypeptide chain for the preparation of fusion proteins, without adversely affecting ligand binding.

The invention therefore also relates to fusion proteins of muteins of the bilin-binding protein, in which an enzyme, another protein or a protein domain, a signal sequence and/or an affinity peptide is fused to the amino terminus of the polypeptide in an operable manner. The invention yet further relates to fusion proteins of bilin-binding protein muteins or of fusion proteins having the amino terminus of biling-binding porotein muteins, in which an enzyme, another protein or a protein domain, a targeting sequence and/or an affinity peptide is fused to the carboxy terminus of the polypeptide in an operable manner.

A preferred enzyme for constructing the fusion proteins of the invention is bacterial alkaline phosphatase (Sowadski et al., J. Mol. Biol. 186 (1985) 417-433) which may be attached either at the N terminus or at the C terminus of a mutein of the bilin-binding protein. In addition, such a fusion protein may carry a signal sequence such as, for example, OmpA or PhoA, which effect secretion of said fusion protein into the *E. coli* periplasm where the disulfide bonds may form efficiently in the polypeptide chain. Furthermore, it may be equipped with an affinity peptide such as, for example, Strep tag II, which allows easy purification of said fusion protein. The specific fusion proteins of the invention are described in the examples. An advantage of a fusion protein of this kind is its ability to catalyze directly a chromogenic, fluorogenic or chemiluminescent detection reaction, which

simplifies its use for detection of the digoxigenin group.

Another advantage of using alkaline phosphatase for constructing fusion proteins of the invention is the fact that this enzyme forms a stable homodimer and, consequently, confers the property of bivalence on the bilin-binding protein mutein as part of the fusion protein. In this way, binding of the digoxigenin group may result in avidity effect which increases detection sensitivity. Such an avidity effect can be expected in particular if the digoxigenin-labeled molecule is adsorbed to a solid phase, is present in oligomeric or membrane-bound form or is conjugated with a plurality of digoxigenin groups. Analogously, other homodimeric enzymes are suitable for preparing bivalent fusion proteins containing the inventive muteins of the bilin-binding protein.

Apart from bacterial alkaline phosphatase, it is also possible to use phosphatases from eukaryotic organisms, such as, for example, calf intestine phosphatase (CIP), for preparing fusion proteins of the invention. Said phosphatases are frequently distinguished by higher enzymatic activity (Murphy and Kantrowitz, Mol. Microbiol. 12 (1994), 351-357), which may result in higher detection sensitivity. It is also possible to use mutants of bacterial alkaline phosphatase, which have improved catalytic activity (Mandecki et al., Protein Eng. 4 (1991), 801-804), for constructing fusion proteins of the invention. Other enzymes known to the skilled worker which catalyze chromogenic, fluorogenic or chemiluminescent reactions, such as, for example, ∃-galactosidase or horseradish peroxidase, are also suitable for preparing fusion proteins of the invention. Moreover, all these enzymes may likewise be employed for labeling muteins of the bilin-binding protein by conjugating them, for example by using

common coupling reagents, with the mutein obtained separately or a fusion protein of the mutein.

In another aspect, the present invention relates to a nucleic acid which comprises a sequence coding for a mutein or a fusion protein of a mutein of the bilin-binding protein. This nucleic acid may be part of a vector which contains operatively functional areas for expressing the nucleic acid. A large number of suitable vectors is known from the prior art and is not described in detail here. Operatively functional areas are those elements which allow, assist, facilitate and/or increase transciption and/or subsequent processing of an mRNA. Examples of elements of this kind include promoters, enhancers, transcription initiation sites and transcription termination sites, translation initiation sites, polyadenylation signals, etc.

The nucleic acid of the invention or its surrounding areas may be such that biosynthesis of the polypeptide takes place in the cytosol, the polypeptide sequence being preceded, where appropriate, by a starting methionine. In a preferred embodiment, however, an N-terminal signal sequence is used, in particular the OmpA or PhoA signal sequence, in order to direct the polypeptide of the invention into the *E. coli* periplasm where the signal sequence is cleaved off by the signal peptidase and the polypeptide chain is able to fold with oxidative formation of the disulfide bonds. Eukaryotic signal sequences may be used in order to secrete the polypeptide of the invention in a eukaryotic host organism. In principle, both prokaryotic, preferably *E. coli*, and eukaryotic cells such as, for example, yeasts are considered for expression of the nucleic acid of the invention.

In yet another aspect, the present invention relates to a method for preparing an inventive mutein or fusion

protein of a mutein of the bilin-binding protein, which method is characterized in that the nucleic acid coding for the mutein or the fusion protein of a mutein of the bilin-binding protein is expressed in a bacterial or eukaryotic host cell and the polypeptide is obtained from the cell or the culture supernatant. For this purpose, normally a suitable host cell is first transformed with a vector which comprises a nucleic acid coding for a polypeptide of the invention. The host cell is then cultured under conditions under which the polypeptide is biosynthesized, and the polypeptide of the invention is obtained.

With respect to the preparation method, it must be taken into account that the inventive muteins of the bilin-binding protein have two structural disulfide bonds and that additional disulfide bonds may be present in corresponding fusion proteins. The formation of said disulfide bonds, which takes place during protein folding, is normally ensured if the polypeptide of the invention is directed with the aid of a suitable signal sequence into a cell compartment containing an oxidizing thiol/disulfide redox medium, for example into the bacterial periplasm or the lumen of the endoplasmic reticulum of a eukaryotic cell. In this connection, the polypeptide of the invention can be liberated by cell fractionation or obtained from the culture supernatant. It is possible, where appropriate, to increase the folding efficiency by overproducing protein disulfide isomerases, for example *E. coli* DsbC protein, or auxiliary folding proteins.

On the other hand, it is possible to produce a polypeptide of the invention in the cytosol of a host cell, preferably *E. coli*. The said polypeptide may then be obtained, for example, in the form of inclusion bodies and then be renatured *in vitro*. Depending on the intended use, the protein can be purified by means of various methods known to the skilled worker. A suitable

method for purifying the inventive muteins of the bilin-binding protein is, for example, affinity chromatography using a column material which carries digoxigenin groups. In order to purify fusion proteins of the muteins of the bilin-binding protein, it is possible to utilize the affinity properties of the fusion protein, which are known from the prior art, for example those of the Strep tag or the Strep tag II (Schmidt and Skerra, J. Chromatogr. A 676 (1994), 337-345; Voss and Skerra, Protein Eng. 10 (1997), 975-982), those of the albumin binding domain (Nygren et al., J. Mol. Recogn. 1 (1988), 69-74) or those of alkaline phosphatase (McCafferty et al., Protein Eng. 4 (1991) 955-961). The fact that the muteins of the bilin-binding protein consist only of a single polypeptide chain is advantageous for the methods for preparing the polypeptides of the invention, since no care needs to be taken either of the need for synthesizing a plurality of various polypeptide chains within a cell simultaneously or of different polypeptide chains associating with one another in a functional manner.

The practical application possibilities for the inventive muteins of the bilin-binding protein essentially correspond to those for conventional antibodies or antibody fragments with binding affinity for digoxigenin. Accordingly, the invention also relates to the use of a mutein of the invention or of a fusion protein of a mutein of the bilin-binding protein in a method for detecting, determining, immobilizing or removing digoxigenin or conjugates of digoxigenin with proteins, nucleic acids, carbohydrates, other biological or synthetic macromolecules or low molecular weight chemical compounds.

The inventive muteins of the bilin-binding protein or their fusion proteins can be used in detection methods essentially in a manner analogous to corresponding detection methods known for anti-digoxigenin antibodies

and also fragments and so-called conjugates thereof. In a further aspect, the present invention therefore relates to a method for detecting the digoxigenin group, in which method a mutein of the bilin-binding protein or a fusion protein of a mutein of the bilin-binding protein is contacted with digoxigenin or with digoxigenin conjugates under conditions suitable for effecting binding of the mutein to the digoxigenin group and the mutein or the fusion protein of the mutein is determined.

For this purpose, the mutein may be labeled directly, for example by covalent coupling. It is, however, also possible to use indirect labeling, for example by means of labeled antibodies against the bilin-binding protein or muteins thereof or against domains of fusion proteins of these muteins. The use of inventive fusion proteins containing an enzyme, for example alkaline phosphatase, instead of a labeled mutein of the bilin-binding protein is particularly advantageous. In this case, it is possible to design the determination method with a particularly small number of process steps, it being possible to utilize directly, for example, the ability of the enzyme as part of the fusion protein to catalyze a chromogenic, fluorogenic or luminescent detection reaction. Here, the fact that such fusion proteins are readily available is a particular advantage compared with corresponding fusion proteins of conventional antibodies. Utilization of the abovedescribed avidity effect in the case of an oligomeric fusion protein is another advantage in such a method.

It is possible to carry out a method for determining the digoxigenin group, for example, qualitatively for detecting nucleic acids conjugated with the digoxigenin group in Southern or Northern blots or proteins conjugated with the digoxigenin group in Western blots. A determination method may also be carried out

quantitatively for detecting proteins conjugated with the digoxigenin group in an ELISA. In addition, a determination method of the invention is also suitable for indirect detection of proteins not conjugated with digoxigenin or of other molecules by using a binding protein which is directed against the protein or molecule, for example an antibody or its fragment, and which is conjugated with the digoxigenin group. Indirect detection of the nucleic acids not conjugated with digoxigenin is also possible by using a gene probe which hybridizes with said nucleic acid and which is conjugated with the digoxigenin group. An application in medical diagnostics or therapy results in addition from determination of digoxigenin, digoxin, digitoxin or digitoxigenin, without these ligands having to be conjugated with another molecule.

The muteins of the invention or fusion proteins thereof may also be used for immobilizing a molecule conjugated with the digoxigenin group. This immobilization is preferably carried out on solid phases coated with the muteins or their fusion proteins, such as, for example, microtiter plates, immunosticks, microbeads made of organic, inorganic or paramagnetic materials, or sensor surfaces.

Correspondingly, it is likewise possible to use the muteins of the invention or fusion proteins thereof for removing digoxigenin, digoxin, digitoxin or digitoxigenin, or a molecule conjugated with one of these compounds. In this case, in addition to the solid phases mentioned, column materials are also considered for coating with the muteins or their fusion proteins. Preferably, said coating is carried out on suitable column materials by coupling by means of chemically reactive groups. Column materials coated in this way may be used for removing substances conjugated with digoxigenin groups and also, where appropriate,

complexes of such substances with other molecules from a solution.

Thus, it is possible, for example, to remove antigens from a solution by adding antibodies to the solution, which are directed against the antigens and conjugated with the digoxigenin group, and contacting the resulting solution with said column material under conditions under which a complex between the digoxigenin groups and an inventive mutein of the bilin-binding protein or its fusion protein is formed. Following such a removal, it is also possible, where appropriate, to elute the substance conjugated with digoxigenin. This elution may be carried out by competition with digoxin, digoxigenin, digitoxin or digitoxigenin and also, for example, by lowering or increasing the pH of the solution. In a competitive elution it is possible to utilize in an advantageous manner the higher binding affinity of the muteins of the invention for digitoxigenin or digitoxin compared with the digoxigenin group. In this way it is possible to isolate or purify a substance conjugated with digoxigenin.

The invention is further illustrated by the following examples and attached drawings, in which:

Fig. 1 represents in each case a fluorescent titration of the Strep tag II-fused mutein DigA16 with the ligands digoxigenin, digitoxigenin and ouabain;

Fig. 2 depicts diagrammatically the expression vectors pBBP27 (A) and pBBP29 (B) for preparing fusion proteins of mutein DigA16 with alkaline phosphatase;

Fig. 3 demonstrates quantitative detection of biomolecules conjugated with digoxigenin

groups by fusion proteins of mutein DigA16 with alkaline phosphatase in an ELISA;

Fig. 4 shows qualitative detection of biomolecules conjugated with digoxigenin groups by fusion proteins of mutein DigA16 with alkaline phosphatase on a Western blot.

Fig. 1 shows the graphic representation of results from Example 3 in which different concentrations of the steroids digoxigenin (squares), digitoxigenin (circles) and ouabain (rhomboids) were added to a 1 µM solution of mutein DigA16. The particular protein fluorescence intensities were measured at an excitation wavelength of 295 nm and an emission wavelength of 345 nm and plotted as a function of the actual total steroid concentration in the particular reaction mixture. Finally, the data points were fitted to a regression curve by means of nonlinear regression.

Fig. 2 shows a drawing of the expression vectors pBBP27 (A) and pBBP29 (B). pBBP27 codes for a fusion protein of bacterial alkaline phosphatase with its own signal sequence, a peptide linker having the sequence Pro-Pro-Ser-Ala, the mutein DigA16 and also the Strep tag II affinity tag. The corresponding structural gene is followed by the *dsbC* structural gene (including its ribosomal binding site) from *E. coli* (Zapun et al., Biochemistry 34 (1995), 5075-5089) as second cistron. The artificial operon formed in this way is under joint transcriptional control of the tetracyclin promoter/operator ($tet^{p/o}$) and ends at the lipoprotein transcription terminator (t_{lpp}). Further vector elements are the origin of replication (ori), the intergenic region of filamentous bacteriophage f1 (f1-IG), the ampicillin resistance gene (bla) coding for ∃-lactamase and the tetracyclin repressor gene (tetR). pBBP29 codes for a fusion protein of the OmpA signal sequence, the mutein DigA16, the Strep tag II affinity tag, a peptide

linker consisting of five glycine residues, and bacterial alkaline phosphatase without its N-terminal amino acid arginine. The vector elements outside this region are identical to vector pBBP27.

Fig. 3 shows a graphic representation of the data from Example 4 in which digoxigenin groups were detected quantitatively with the aid of mutein DigA16 fusion proteins as gene products of vectors pBBP27 (closed symbols) and pBBP29 (open symbols). Here, the digoxigenin groups were coupled on the one hand to bovine serum albumin (BSA, squares) or, on the other hand, to egg albumin (ovalbumin, triangles). The control data shown are those obtained when using underivatized bovine serum albumin and the fusion protein encoded by pBBP27 (open circles). The enzymic activity corresponding to the particular bound fusion protein was monitored spectrophotometrically at 405 nm on the basis of p-nitrophenyl phosphate hydrolysis. Curve fitting was carried out by nonlinear regression with the aid of the Kaleidagraph computer program (Abelbeck Software) by means of the equation

$$[P \bullet L]=[L]_t[P]_t/(K_d+[P]_t).$$

Here, $[P]_t$ corresponds to the total fusion protein concentration used in the particular microtiter plate well. $[P \bullet L]$ is determined on the basis of the enzymic activity of alkaline phosphatase. The total concentration of digoxigenin groups $[L]_t$, constant within a concentration series, per well and the dissociation constant K_d were fitted as parameters by nonlinear regression.

Fig. 4 shows the result of a Western blot experiment from Example 4 for qualitative detection of biomolecules conjugated with digoxigenin groups by means of the mutein DigA16 fusion proteins encoded by pBBP27 (lanes 1 and 2) and pBBP29 (lanes 3 and 4). For

comparison, a 15% strength SDS polyacrylamide gel of the biomolecules, stained with Coomassie Brilliant Blue, is likewise shown (lanes 5 and 6). Here, a mixture of 0.5 µg of underivatized BSA, underivatized ovalbumin and underivatized RNaseA was fractionated in each case in lanes 1, 3 and 5. A mixture of 0.5 µg of BSA coupled to digoxigenin groups, ovalbumin coupled to digoxigenin groups and RNaseA coupled to digoxigenin groups was fractionated in each case in lanes 2, 4 and 6.

Examples

Unless stated otherwise, the genetic engineering methods familiar to the skilled worker, as described, for example, in Sambrook et al. (Molecular Cloning. A Laboratory Manual (1989), Cold Spring Harbor Press) were used.

Example 1

Preparation of a library for muteins of the bilin-binding protein, phagemid presentation and selection of a mutein with binding affinity for digoxigenin

A library for muteins of the bilin-binding protein was prepared by subjecting the amino acid sequence positions of said bilin-binding protein, 34, 35, 36, 37, 58, 60, 69, 88, 90, 93, 95, 97, 114, 116, 125 and 127, to a concerted mutagenesis in multiple steps with the aid of the polymerase chain reaction (PCR). The PCR reactions were initially carried out in two separate amplification steps in a volume of in each case 50 µl, and 10 ng of pBBP20 phasmid DNA (SEQ ID NO: 1) as matrix and in each case 25 pmol of two primers (SEQ ID NO. 2 and SEQ ID NO. 3 in one mixture and SEQ ID NO. 4 and SEQ ID NO. 5 in a second mixture) which had been synthesized according to the generally known phosphoramidite method were used.

Furthermore, the reaction mixture contained 5 µl of 10xTaq buffer (100 mM Tris/HCl pH 9.0, 500 mM KCl, 1% v/v Triton X-100), 3 µl of 25 mM $MgCl_2$ and 4 µl of dNTP mix (2.5 mM dATP, dCTP, dGTP, dTTP). After filling up with water, the mixture was overlaid with mineral oil and heated to 94°C in a programmable thermostating block for 2 min. Then 2.5 u of Taq DNA polymerase (5 u/µl, Promega) were added and 20 temperature cycles of 1 min at 94°C, 1 min at 60°C and 1.5 min at 72°C were carried out, followed by an incubation at 60°C for 5 min. The desired amplification products were isolated via preparative agarose gel electrophoresis from low melting point agarose Jetsorb (Gibco BRL), using the DNA extraction kit (Genomed) according to the manufacturer's instructions.

A relevant section of the pBBP20 nucleic acid sequence is shown together with the encoded amino acid sequence as SEQ ID NO. 1 in the sequence listing. The section starts with a hexanucleotide sequence which was obtained by ligating an *Xba*I overhang with an *Spe*I overhang complementary thereto and ends with the *Hind*III cleavage site. The vector elements outside this region are identical to vector pASK75 whoses complete nucleotide sequence is stated in the publication DE 44 17 598 A1.

The subsequent amplification step was carried out in a 100 µl mixture, and in each case approx. 6 ng of the two isolated fragments as matrix, 50 pmol of each of the two primers SEQ ID NO. 6 and SEQ ID NO. 7 and also 1 pmol of oligodeoxynucleotide SEQ ID NO. 8 were used. The remaining components of the PCR mixture were added in twice the amount, as in the preceding amplification steps. The PCR was carried out in 20 temperature cycles of 1 min at 94°C, 1 min at 55°C, and 1.5 min at 72°C, followed by a final incubation at 60°C for 5 min. The fragment obtained was again isolated by preparative agarose gel electrophoresis.

Said fragment which represented the mutein library in the form of a mixture of nucleic acids was cloned by cutting it first with the restriction enzyme *Bst*XI (New England Biolabs) according to the manufacturer's instructions. The nucleic acid fragment obtained (335 base pairs, bp) was purified again by means of preparative agarose gel electrophoresis. Analogously, pBBP20 vector DNA was cut with *Bst*XI and the larger of the two fragments (3971 bp) was isolated.

For ligation, 0.93 µg (4.2 pmol) of the PCR fragment and 11 µg (4.2 pmol) of the vector fragment were incubated in the presence of 102 Weiss units of T4 DNA ligase (New England Biolabs) in a total volume of 500 µl (50 mM Tris/HCl pH 7.8, 10 mM MgCl, 10 mM DTT, 1 mM ATP, 50 µg/ml BSA) at 16°C for two days. The DNA was then precipitated by adding 10 µg of yeast tRNA (Boehringer Mannheim), 25 µl of 5 M ammonium acetate and 100 µl of ethanol to in each case 24 µl of the ligation mixture. Incubation at -20°C for 3 days was followed by centrifugation (25 min, 16000 g, 4°C). The precipitate was washed in each case with 200 µl of ethanol (70% v/v, -20°C) and dried under reduced pressure. Finally, the DNA was taken up in 43.6 µl of TE/10 (1 mM Tris/HCl pH 8.0, 0.1 mM EDTA). The DNA concentration of the solution obtained was estimated by analytical agarose gel electrophoresis on the basis of the fluorescence intensity of the bands stained with ethidium bromide in comparison with a DNA size standard of known concentration.

Electrocompetent cells of the *E. coli* K12 strain XL1-Blue (Bullock et al., BioTechniques 5 (1987), 376-379) were prepared according to the methods described by Tung and Chow (Trends Genet. 11 (1995), 128-129) and by Hengen (Trends Biochem. Sci. 21 (1996), 75-76). 1 l of LB medium was adjusted to an optical density at 600 nm, OD_{600} = 0.08 by adding a stationary XL1-Blue overnight culture and incubated in a 3 l Erlenmeyer

flask at 200 rpm and 26°C. After reaching OD_{600} = 0.6, the culture was cooled on ice for 30 min and then centrifuged at 4000 g and 4°C for 15 min. The cell sediment was washed twice with in each case 500 ml of ice cold 10% w/v glycerol and finally resuspended in 2 ml of ice cold GYT medium (10% w/v glycerol, 0.125% w/v yeast extract, 0.25% w/v tryptone).

Electroporation was carried out by using the Easyjac T Basic system (EquiBio) with the corresponding cuvettes (electrode distance 2 mm). All operational steps were carried out in a cold room at 4°C. 5 to 6 µl of the above-described DNA solution (245 ng/µl) were in each case mixed with 40 µl of the cell suspension, incubated on ice for 1 min and then transferred into the cuvette. After electroporation, the suspension was immediately diluted in 2 ml of fresh ice-cold SOC medium (2% w/v tryptone, 0.5% w/v yeast extract, 10 mM NaCl, 10 mM $MgSO_4$, 10 mM $MgCl_2$) and agitated at 37°C and 200 rpm for 60 min. The cells were then sedimented at 3600 g for in each case 2 min, resuspended in 1 ml of LB medium containing 100 µg/ml of ampicillin (LB/Amp) and plated out at 200 µl each on agar plates (140 mm in diameter) with LB/Amp medium. Using a total of 10.7 µg of the ligated DNA in eight electroporation mixtures produced in this way $3.73 \cdot 10^8$ transformants which were distributed on 40 agar plates.

After incubation at 32°C for 14 h, the colonies obtained in this way were scraped off the agar plates with the addition of in each case 10 ml of 2xYT/Amp medium, transferred to a sterile Erlenmeyer flask and agitated at 37°C, 200 rpm for 20 min to complete resuspension. 50 ml of 2xYT/Amp medium prewarmed to 37°C were inoculated with 2.88 ml of said suspension so that the cell density was 1.0 OD_{550}. This culture was incubated at 37°C, 160 rpm for 6 h to reach a stationary cell density, and phasmid DNA was isolated with the aid of the plasmid Midi kit (Qiagen) according

to the manufacturer's instructions. Finally, the DNA was taken up in 100 µl of TE (10 mM Tris/HCl pH 8.0, 1 mM EDTA) and stored at 4°C for further use.

In order to prepare a library of recombinant phagemids (Kay et al., Phage Display of Peptides and Proteins - A Laboratory Manual (1996), Academic Press) which carry the muteins of the bilin-binding protein as a fusion with the truncated coat protein pIII, the phasmid DNA obtained in this way was used for transformation of electrocompetent cells of *E. coli* XL1-Blue. Electroporation was carried out as described above with the aid of the Easyjec T Basic system. In a total of 13 mixtures, 40 µl of the cell suspension of electrocompetent cells were in each case transformed with in each case 2 µg of the DNA in a volume of 5 µl. After electroporation, the cell suspension obtained from each mixture was diluted immediately in 2 ml of fresh ice-cold SOC medium and agitated at 37°C and 200 rpm for 60 min.

The mixtures were combined (volume = 26 ml) and 74 ml of 2xYT medium and 100 µl of ampicillin (stock solution 100 mg/ml, final concentration 100 mg/l) were added. The total number of transformants obtained was estimated at $1.1 \cdot 10^{10}$ by plating out 100 µl of a $1:10^5$ dilution of the obtained suspension on agar plates containing LB/Amp medium. After incubation at 37°C and 160 rpm for 60 min, the culture was infected with 500 µl of VCS-M13 helper phage ($1.1 \cdot 10^{12}$ pfu/ml, Stratagene) and agitated at 37°C, 160 rpm for a further 60 min. Subsequently, 200 µl of kanamycin (stock solution 35 mg/ml, final concentration 70 mg/l) were added, the incubator temperature was lowered to 26°C and, after 10 min, anhydrotetracyclin (50 µl of a 50 µg/ml stock solution in dimethylformamide, final concentration 25 µg/l) was added to induce gene expression. Finally, the phagemids were produced by incubating the culture at 26°C, 160 rpm for 7 h.

The cells were removed by centrifugation of the culture (15 min, 12000 g, 4°C). The supernatant containing the phagemid particles was sterile-filtered (0.45 µm), mixed with 1/4 volume (25 ml) of 20% w/v PEG 8000, 15% w/v NaCl and incubated at 4°C overnight. After centrifugation (20 min, 18000 g, 4°C), the precipitated phagemid particles were dissolved in a total of 4 ml of cold PBS (4 mM KH_2PO_4, 16 mM Na_2HPO_4, 115 mM NaCl, pH 7.4). The solution was incubated on ice for 30 min and distributed into four 1.5 ml reaction vessels at equal volumes. After removing undissolved components by centrifugation (5 min, 18500 g, 4°C), the supernatant was transferred in each case to a new reaction vessel.

The phagemid particles were again precipitated by mixing with 1/4 volume (in each case 0.25 ml per reaction vessel) of 20% w/v PEG 8000, 15% w/v NaCl and incubating on ice for 60 min. After centrifugation (20 min, 18500 g, 4°C), the supernatant was removed and the precipitated phagemid particles were each dissolved in 0.5 ml of PBS. After incubation on ice for 30 min, centrifugation (5 min, 18500 g, 4°C) was repeated to clarify the solution. The supernatant containing the phagemid particles (between $1 \cdot 10^{12}$ and $5 \cdot 10^{12}$ cfu/ml) was then used for affinity concentration.

The recombinant phagemids presenting the muteins of the bilin-binding protein were affinity-concentrated using Immuno Sticks (NUNC). These were coated overnight with 800 µl of a conjugate (100 µg/ml) of ribonuclease A (RNaseA) and digoxigenin in PBS.

The conjugate was prepared by adding 1.46 µmol (0.96 mg) of digoxigenin-3-O-methylcarbonyl-ε-amino-caproic acid N-hydroxysuccinimide ester (DIG-NHS, Boehringer Mannheim) in 25 µl of DMSO in µl steps and with constant mixing to 0.73 µmol (10 mg) of RNaseA (Fluka) in 1 ml of 5% w/v sodium hydrogen carbonate. The mixture was incubated with stirring at room

temperature (RT) for 1 h. Excess reagent was then removed from the RNaseA conjugate by means of a PD-10 gel filtration column (Pharmacia) according to the manufacturer's instructions.

Unoccupied binding sites on the Immuno Stick surface were saturated by incubation with 1.2 ml of 2% w/v BSA in PBST (PBS with 0.1% v/v Tween 20) at RT for 2 h. After three short washes with in each case 1.2 ml of PBST, the Immuno Stick was incubated in a mixture of 250 µl of phagemid solution and 500 µl of blocking buffer (2% w/v BSA in PBST) at RT for 1 h.

Unbound phagemids were removed by stripping off the solution and washing the Immuno Stick eight times with in each case 950 µl of PBST for 2 min. Finally, adsorbed phagemids were competitively eluted during a 15 minute incubation of the Immuno Stick with 950 µl of a 2 mM solution of digoxigenin in PBS (0.742 mg of digoxigenin (Fluka) were to this end dissolved in 19.2 µl of DMF and added to 930.8 µl of PBS).

The phagemids were propagated by heating 950 µl of solution of the elution fraction obtained (between 10^6 and 10^8 colony-forming units, depending on the selection cycle) briefly to 37°C, mixing the solution with 4 ml of an exponentially growing culture of *E. coli* XL1-Blue (OD_{550} = 0.5) and incubated at 37°C, 200 rpm for 30 min. The phagemid-infected cells were then sedimented (2 min, 4420 g, 4°C), resuspended in 800 µl of fresh 2xxYT medium and plated out on four agar plates containing LB/Amp medium (140 mm in diameter). After incubation at 32°C for 14 h, the colonies obtained in this way were scraped off the agar plates with the addition of in each case 10 ml of 2xYT/Amp medium, transferred to a sterile Erlenmeyer flask and agitated at 37°C, 200 rpm for 20 min to complete resuspension.

Production and affinity concentration of phagemid particles were repeated by inoculating 50 ml of 2xYT/Amp medium prewarmed to 37°C with 0.2 to 1 ml of said suspension so that the cell density was 0.08 OD_{550}. This culture was incubated at 37°C, 160 rpm to a cell density of OD_{550} = 0.5, infected with 250 µl of VCS-M13 helper phage ($1.1 \cdot 10^{12}$ pfu/ml, Stratagene), and the procedure was continued as already described above.

The phagemids obtained from the first affinity concentration were used to carry out a series of eight further concentration cycles using Immuno Sticks which had been freshly coated with the digoxigenin-RNaseA conjugate. The phagemids obtained after the last concentration cycle were again used for infecting *E. coli* XL1-Blue. The mixture of the colonies obtained was scraped off the agar plates using 2xYT/Amp medium and resuspended, as described above. This cell suspension was used to inoculate 50 ml of 2xYT/Amp medium, and the phasmid DNA was isolated using the QIAprep Spin Miniprep kit (QIAGEN) according to the manufacturer's instructions.

In order to be able to produce the muteins of the bilin-binding protein as a fusion protein with the Strep tag II and the albumin-binding domain, the gene cassette between the two *Bst*XI cleavage sites was subcloned from vector pBBP20 into vector pBBP22. A relevant section of the pBBP22 nucleic acid sequence is represented, together with the encoded amino acid sequence, as SEQ ID NO. 9 in the sequence listing. The section starts with the *Xba*I cleavage site and ends with the *Hind*III cleavage site. The vector elements outside this region are identical to vector pASK75.

For this purpose, the DNA isolated from the mixture of the *E. coli* colonies was cut with restriction enzyme *Bst*XI, and the smaller of the two fragments (335 bp) was purified by preparative agarose gel electrophoresis

as described above. In the same manner, pBBP22 vector DNA was cut with *Bst*XI and the larger of the two fragments (3545 bp) was isolated.

1.5 Weiss units of T4 DNA ligase (Promega) were added to 50 fmol of each of the two DNA fragments in a total volume of 20 µl (30 mM Tris/HCl pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP) and the mixture was incubated for ligation at 16°C overnight. 5 µl of this ligation mixture were used to transform 200 µl of competent cells of *E. coli* strain TG1-F^- according to the $CaCl_2$ method (Sambrook et al., supra), and 2.2 ml of a cell suspension were obtained.

The transformants were then screened for production of muteins with binding activity for the digoxigenin group by means of a colony screening assay. For this purpose, a cut-to-fit hydrophilic PVDF membrane (Millipore, type GVWP, pore size 0.22 µm) was marked at one position and placed on an LB/Amp agar plate. 150 µl of the cell suspension from the transformation mixture were plated out evenly on said membrane, and approx. 500 colonies were obtained. The plate was incubated in an incubator at 37°C for 7.5 h until the colonies were approx. 0.5 mm in diameter.

In the meantime, a hydrophobic membrane (Millipore, Immobilon P, pore size 0.45 µm) which had likewise been cut to fit was wetted with PBS according to the manufacturer's instructions and subsequently gently agitated in a solution of 10 mg/ml of human serum albumin (HSA, Sigma) in PBS at RT for 4 h. Remaining binding sites on the membrane were saturated by incubation with 3% w/v BSA, 0.5% v/v Tween 20 in PBS at RT for 2 h. The membrane was washed with 20 ml of PBS for two times 10 min and then gently agitated in 10 ml of LB/Amp medium to which 200 µg/l of anhydrotetracyclin had been added for 10 min. Said membrane was then marked at one position and placed on

a culture plate with LB/Amp agar which additionally contained 200 µg/l of anhydrotetracyclin.

The previously obtained hydrophilic membrane on which colonies had grown was then placed onto the hydrophobic membrane such that the two markings coincided. The culturing plate with the two membranes was incubated at 22°C for 15 h. During this phase, the particular muteins were secreted by the colonies as fusion proteins and immobilized on the lower membrane by means of complex formation between the albumin-binding domain and the HSA.

Subsequently, the upper membrane containing the colonies was transferred to a fresh LB/Amp agar plate and stored at 4°C. The hydrophobic membrane was removed, washed with 20 ml of PBST for three times 10 min and then incubated in 10 ml of a 10 µg/ml solution of a conjugate of BSA with digoxigenin in PBST for 1 h.

The conjugate of BSA (Sigma) and digoxigenin was prepared by adding a solution of 3.0 µmol (1.98 mg) of DIG-NHS in 25 µl of DMSO in µl steps and with constant mixing to 300 nmol (19.88 mg) of BSA (Sigma) in 1.9 ml of 5% w/v sodium hydrogen carbonate. The mixture was incubated with stirring at RT for 1 h and excess reagent was removed from the BSA conjugate by means of a PD-10 gel filtration column according to the manufacturer's instructions.

In order to detect bound digoxigenin-BSA conjugate, the membrane was incubated, after washing twice in 20 ml of PBST, with 10 ml of anti-digoxigenin Fab-alkaline phosphatase conjugate (Boehringer Mannheim, diluted 1:1000 in PBST) for 1 h. The membrane was then washed twice with 20 ml PBST and twice with 20 ml of PBST for in each case 5 min and gently agitated in AP buffer (0.1 M Tris/HCl pH 8.8, 0.1 M NaCl, 5 mM $MgCl_2$) for

10 min. For the chromogenic detection reaction, the membrane was incubated in 10 ml of AP buffer to which 30 µl of 5-bromo-4-chloro-3-indolyl phosphate, p-toluidinium salt (BCIP, Roth, 50 µg/ml in dimethylformamide) and 5 µl of Nitro Blue Tetrazolium (NBT, Sigma, 75 µg/ml in 70% v/v dimethylformamide) had been added, until at the positions of some of the colonies distinct color signals became visible. In this way, digoxigenin-binding activity of the bilin-binding protein muteins which had been produced in the form of fusion proteins with Strep tag and ABD by said colonies was detected.

Four colonies from the upper membrane, which caused a distinct color signal, were used for preparing cultures in LB/Amp medium of 4 ml in volume. Their plasmid DNA was isolated with the aid of the JETquick Plasmid Miniprep Spin kit (Genomed) according to the manufacturer's instructions, and the gene section coding for the mutein was subjected to sequence analysis. Sequence analysis was carried out with the aid of the T7 sequencing kit (Pharmacia) according to the manufacturer's instructions by using oligodeoxynucleotides SEQ ID NO. 10 and SEQ ID NO. 11. It was found in the process that all four plasmids studied carried the same nucleotide sequence. The corresponding gene product was denoted by DigA (SEQ ID NO. 12). The DigA nucleotide sequence was translated into the amino acid sequence and is represented in the sequence listing.

Example 2

Partial random mutagenesis of the DigA mutein and selection of muteins with improved binding affinity for digoxigenin

In order to improve the affinity between the DigA mutein and digoxigenin, which was determined as

295 ± 36 nM according to Example 3, the 6 amino acid positions 28, 31 and 34-37 in DigA were selected for a more substantial partial random mutagenesis.

Said positions were mutated by carrying out the PCR using a degenerated oligodeoxynucleotide primer. The amplification reaction was carried out in a total volume of 100 µl, with 2 ng of the vector pBBP22 plasmid DNA coding for DigA (SEQ ID NO. 12) being used as matrix. The reaction mixture contained 50 pmol of the two primers SEQ ID NO. 13 and SEQ ID NO. 7 and also the other components according to the method described in Example 1. The PCR was carried out in 20 temperature cycles of 1 min at 94°C, 1 min at 65°C, and 1.5 min at 72°C, followed by a final incubation at 60°C for 5 min. The DNA fragment obtained was isolated by preparative agarose gel electrophoresis and then cut with *Bst*XI according to the manufacturer's instructions. The resulting DNA fragment of 335 bp in length was again purified by preparative agarose gel electrophoresis.

PBBP24 vector DNA was cut with *Bst*XI accordingly and the 4028 bp fragment obtained was isolated. A relevant section of the pBBP24 nucleic acid sequence is represented, together with the encoded amino acid sequence, as SEQ ID NO. 14 in the sequence listing. The section starts with the *Xba*I cleavage site and ends with the *Hind*III cleavage site. The vector elements outside this region are identical to vector pASK75. PBBP24 is virtually identical with pBBP20, and the BBP gene has been inactivated by means of appropriately introduced stop codons.

1.3 µg of the cleaved DNA fragment from the PCR and 16.0 µg of the pBBP24 fragment were incubated for ligation in the presence of 120 Weiss units of T4 DNA ligase (New England Biolabs) in a total volume of 600 µl (50 mM Tris/HCl pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 50 µg/ml BSA) at 16°C for 18 h. The DNA was

then precipitated by adding 10 µg of yeast tRNA (Boehringer Mannheim), 25 µl of 5 M ammonium acetate and 100 µl of ethanol to in each case 24 µl of the ligation mixture. Incubation at -20°C for two weeks was followed by centrifugation (20 min, 16000 g, 4°C). The precipitate was washed in each case with 150 µl of ethanol (70% v/v, -20°C) and dried under reduced pressure. Finally, the DNA was taken up in 80 µl of TE/10.

E. coli XL1-Blue cells were transformed with the ligated DNA by electroporation according to the procedure described in Example 1, with in each case 40 µl of cell suspension of electrocompetent cells being mixed with 5 µl of the DNA solution in 16 mixtures. After electroporation, the cells were immediately diluted in 2 ml of fresh ice-cold SOC medium and agitated at 37°C and 200 rpm for 60 min.

168 ml of 2xYT medium and 200 µl of ampicillin (stock solution 100 mg/ml, final concentration 100 mg/l) were added to the combined suspensions. The total number of transformants obtained was estimated at $1.48 \cdot 10^9$ by plating out 100 µl of a $1:10^4$ dilution of the obtained cell suspension on agar plates. After incubation at 37°C and 160 rpm for 60 min, the transformants were infected with 4 ml of VCS-M13 helper phage ($6.3 \cdot 10^{11}$ pfu/ml, Stratagene) and agitated at 37°C and 160 rpm for a further 30 min. Subsequently, 400 µl of kanamycin (stock solution 35 mg/ml, final concentration 70 mg/l) were added, the incubator temperature was lowered to 26°C and, after 10 min, anhydrotetracyclin (100 µl of a 50 µg/ml stock solution in dimethylformamide, final concentration 25 µg/l) was added to induce gene expression. Finally, the phagemids were produced by incubating the culture at 26°C and 160 rpm for 7 h. The cells were removed and the phagemids purified by precipitation as described in Example 1.

Streptavidin-coated paramagnetic particles (Dynabeads M-280 Streptavidin, Dynal) were used together with a double conjugate of BSA with digoxigenin and biotin for affinity concentration from the library of phagemids which presented the partially mutated DigA mutein.

A double conjugate of BSA with digoxigenin and biotin was prepared by adding 1.5 µmol (0.99 mg) of DIG-NHS in 12.5 µl of DMSO and 1.5 µmol (0.68 mg) of D-biotinoyl-ε-aminocaproic acid N-hydroxysuccinimide ester (Boehringer Mannheim) in 12.5 µl of DMSO in µl steps and with constant mixing to 300 nmol (19.88 mg) of BSA in 1.9 ml of 5% w/v sodium hydrogen carbonate. The mixture was incubated with stirring at RT for 1 h. Excess reagent was removed from the double conjugate via a PD-10 gel filtration column according to the manufacturer's instructions.

Digoxigenin-binding phagemids were concentrated by mixing 40 µl of a 0.5 µM solution of the double conjugate (33.5 µg/ml) in PBS with 260 µl of a solution of the freshly prepared phagemids (between $5 \cdot 10^{11}$ and $5 \cdot 10^{12}$ cfu/ml) and incubated at RT for 1 h so that the complex between the digoxigenin group and the muteins presented by the phagemids was able to form. This was followed by adding 100 µl of a solution of 8% w/v BSA, 0.4% v/v Tween 20 in PBS.

Parallel thereto, 100 µl of the commercially available suspension of paramagnetic particles were washed with three times 100 µl of PBS. Here, the particles were kept suspended for 1 min by rotating the 1.5 ml Eppendorf vessel and then collected at the wall of the Eppendorf vessel with the aid of a magnet, and the supernatant was stripped off. Unspecific binding sites were saturated by incubating the paramagnetic particles with 100 µl of 2% w/v BSA in PBST at RT for 1 h. After removing the supernatant, the mixture of double conjugate and phagemids was added to the paramagnetic

particles, and the particles were resuspended and incubated at RT for 10 min. Finally, free biotin-binding sites of Streptavidin were saturated by adding 10 µl of a 4 µM D-dethiobiotin (Sigma) solution in PBS to the mixture and incubating said mixture at RT for 5 min. This procedure also prevented the Strep tag II as part of the fusion protein of the muteins and the phage coat protein pIII fragment from being able to form a complex with Streptavidin.

Unbound phagemids were removed by washing the paramagnetic particles with eight times 1 ml of fresh PBST with the addition of 1 mM D-dethiobiotin, the particles were collected with the aid of the magnet and the supernatant was stripped off. The bound phagemids were eluted by incubating the resuspended particles in 950 µl of 0.1 M glycine/HCl pH 2.2 for 15 minutes. After collecting the particles on the magnet, the supernatant was again stripped off and this was immediately followed by neutralizing the pH of said solution by addition of 140 µl of 0.5 M Tris.

The phagemids were propagated by mixing the elution fraction obtained, according to the procedure in Example 1, with 4 ml of an exponentially growing culture of *E. coli* XL1-Blue (OD_{550} = 0.5) and incubating at 37°C, 200 rpm for 30 min. The phagemid-infected cells were then sedimented (2 min, 4420 g, 4°C), resuspended in 800 µl of fresh 2xYT medium and plated out on four agar plates containing LB/Amp medium (140 mm in diameter). After incubation at 32°C for 14 h, the colonies obtained in this way were scraped off the agar plates with the addition of in each case 10 ml of 2xYT/Amp medium, transferred to a sterile Erlenmeyer flask and agitated at 37°C, 200 rpm for 20 min to complete resuspension.

Production and affinity concentration of phagemid particles were repeated by inoculating 50 ml of

2xYT/Amp medium prewarmed to 37°C with 0.2 to 1 ml of said suspension so that the cell density was 0.08 OD_{550}. This culture was incubated at 37°C, 160 rpm to a cell density of OD_{550} = 0.5 and infected with 300 µl of VCS-M13 helper phage ($6.3 \cdot 10^{11}$ pfu/ml, Stratagene). The affinity selection was then repeated using the paramagnetic particles and the digoxigenin/biotin double conjugate under the abovementioned conditions. A total of 4 selection cycles were carried out in this way.
The phagemids obtained after the last concentration cycle were again used for infecting *E. coli* XL1-Blue. The mixture of the obtained colonies which had been scraped off the agar plates using 2xYT/Amp medium and had been resuspended, as described above, was used to inoculate 50 ml of 2xYT/Amp medium, and phasmid DNA was isolated using the QIAprep spin miniprep kit (QIAGEN) according to the manufacturer's instructions.

Subsequently, the gene cassette between the two *Bst*XI cleavage sites was subcloned, as in Example 1, from vector pBBP24 into vector pBBP22, and competent cells of *E. coli* strain TG1-F^- were transformed according to the $CaCl_2$ method. Finally, the transformants were, again according to Example 1, screened for production of muteins with binding activity for the digoxigenin group by means of the colony screening assay.

Seven of the colonies showing a strong signal intensity in the colony screening assay were cultured. Their plasmid DNA was isolated with the aid of the plasmid miniprep spin kit (Genomed) according to the manufacturer's instructions, and the gene section coding for the mutein was subjected to sequence analysis as in Example 1. It was found in the process that all plasmids studied had different sequences. After translating the nucleotide sequences into amino acid sequences, six of the seven variants studied had an amber stop codon at amino acid position 28. However,

this stop codon was at least partially suppressed when choosing suitable amber-suppressor strains such as, for example, *E. coli* XL1-Blue or TG1-F^- and instead translated as glutamine. Thus a full-length functional protein was produced both during affinity concentration and in the colony screening assay.

As the only mutein without an amber stop codon among the muteins found, the mutein with SEQ ID NO. 15 was particularly well suited to bacterial production. Consequently, this mutein, also denoted by DigA16, was characterized in more detail with regard to its ability to bind to the digoxigenin group.

Example 3

Production of the DigA and DigA16 muteins and determination of their affinity for digoxigenin and derivatives thereof by fluorescence titration

The bilin-binding protein muteins obtained from the previous examples were preparatively produced by subcloning the coding gene section between the two *Bst*XI cleavage sites from the type pBBP22 vector into the expression plasmid pBBP21. The plasmid thus obtained coded for a fusion protein of the OmpA signal sequence, followed by the mutein and the Strep tag II affinity tag.

A relevant section of the pBBP21 nucleic acid sequence is represented, together with the encoded amino acid sequence, as SEQ ID NO. 16 in the sequence listing. The section starts with the *Xba*I cleavage site and ends with a hexanucleotide which was obtained by ligating a blunt strand end with a filled-up *Hind*III strand end, with the loss of the original *Hind*III cleavage site. The vector elements outside this region are identical to vector pASK75.

For subcloning, the plasmid DNA coding for the relevant mutein was cut with restriction enzyme *Bst*XI, and the smaller of the two fragments (335 bp) was purified by preparative agarose gel electrophoresis as described in Example 1. In the same manner, pBBP21 vector DNA was cut with *Bst*XI, and the larger of the two fragments (4132 bp) was isolated.

1.5 Weiss units of T4 DNA ligase (Promega) were added to 50 fmol of each of the two DNA fragments in a total volume of 20 µl (30 mM Tris/HCl pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP) and the mixture was incubated for ligation at 16°C for 16 h. 5 µl of the ligation mixture were then used to transform *E. coli* JM83 (Yanisch-Perron et al., Gene 33 (1985), 103-119) according to the $CaCl_2$ method, and 2.2 ml of a cell suspension were obtained. 100 µl of this suspension were plated out on an agar plate containing LB/Amp medium and incubated at 37°C for 14 h.

The protein was produced by selecting one of the obtained single colonies, using it to inoculate a 50 ml preculture (LB/Amp medium) and incubating said preculture at 30°C and 200 rpm overnight. 40 ml of the preculture were then transferred by inoculating 2 l of LB/Amp medium in a 5 l Erlenmeyer flask, followed by incubating the culture at 22°C and 200 rpm. At a cell density of OD_{550} = 0.5, gene expression was induced by adding 200 µg/l anhydrotetracyclin (200 µl of a 2 mg/ml stock solution in DMF), followed by agitating at 22°C, 200 rpm for a further 3 h.

The cells were removed by centrifugation (15 min, 4 420 g, 4°C) and, after removing the supernatant, resuspended in 20 ml of periplasm lysis buffer (100 mM Tris/HCl pH 8.0, 500 mM sucrose, 1 mM EDTA) with cooling on ice. After incubation on ice for 30 min, the spheroplasts were removed in two successive centrifugation steps (15 min, 4 420 g, 4°C and 15 min,

30 000 g, 4°C). The periplasmic protein extract obtained in this way was dialyzed against SA buffer (100 mM Tris/HCl pH 8.0, 150 mM NaCl, 1 mM EDTA), sterile-filtered and used for chromatographic purification.

Purification was carried out by means of the Strep tag II affinity tag (Schmidt and Skerra, Protein Eng. 6 (1993), 109-122) fused to the C terminus of the muteins. In the present case, Streptavidinmutein "1" was used (Voss and Skerra, Protein Eng. 10 (1997), 975-982), which was coupled to activated Sepharose (via 5 mg/ml immobilized Streptavidin, based on the bett volume of the matrix).

A chromatography column packed with 2 ml of said material was equilibrated at 4°C and a flow rate of 20 ml/h with 10 ml of SA buffer. The chromatography was monitored by measuring absorption of the eluate at 280 nm in a flow-through photometer. Application of the periplasmic protein extract was followed by washing with SA buffer until the base line was reached. Bound mutein was then eluted with 10 ml of a solution of 2.5 mM D-dethiobiotin (Sigma) in SA buffer. The fractions containing the purified mutein were checked by means of SDS polyacrylamide gel electrophoresis (Fling and Gregerson, Anal. Biochem. 155 (1986), 83-88) and combined. The protein yields were between 200 µg and 800 µg per 2 l culture.

The ligand binding properties of muteins DigA, DigA16 and also of the recombinant bilin-binding protein (SEQ ID NO: 16) were determined by means of the method of fluorescence titration.

In this case, the decrease in intrinsic tyrosine and/or tryptophan fluorescence of the protein forming a complex with the ligand was measured. The measurements were carried out in a fluorimeter, type LS 50 B (Perkin

Elmer) at an excitation wavelength of 295 nm (slit width 4 nm) and an emission wavelength of 345 nm (slit width 6 nm). The ligands used were digoxigenin (Fluka), digoxin (Fluka), digitoxigenin (Fluka), digitoxin (Fluka), testosterone (Sigma), ouabain (Fluka), and 4-aminofluorescein (Fluka). The ligands showed no significant intrinsic fluorescence or absorption at the stated wavelength.

The buffer system used was PBS with the addition of 1 mM EDTA. The solution of the relevant purified mutein was dialyzed four times against this buffer and adjusted to a concentration of 1 µm by dilution. All solutions used were sterile-filtered (Filtropur S 0.45 µm, Sarstedt). The concentration was determined by means of absorption at 280 nm using calculated extinction coefficients of 53 580 M^{-1} cm^{-1} for DigA and DigA16 (Wisconsin Software Package, Genetics Computer Group). For Bbp, the calculated extinction coefficient of 54 150 M^{-1} cm^{-1}, corrected in the presence of guanidinium chloride according to Gill and von Hippel (Anal. Biochem. 182 (1989), 319-326) was used.

For the measurement, 2 ml of the mutein solution were introduced into a quartz cuvette equipped with a magnetic stirrer bar and thermally equilibrated at 25°C in the sample holder of the photometer. Then a total of 40 µl of a 100 µM to 500 µM solution of the ligand in the same buffer were pipetted in steps of from 1 µl to 4 µl. The dilution of the introduced protein solution by altogether no more than 2%, which took place in the process, was not taken into account in the subsequent evaluation of the data. After each titration step, the equilibrium was allowed to form by incubating with stirring for 1 min, and the fluorescence signal was measured as average over 10 s. After subtracting the fluorescence value of the buffer, the signals were normalized to an initial value of 100%.

The thus obtained data of a titration series were fitted by nonlinear regression with the aid of the computer program Kaleidagraph (Abelbeck Software) according to the following formula

$$F=([P]_t-[L]_t-K_d)\frac{f_P}{2}+([P]_t+[L]_t+K_d)\frac{f_{PL}}{2}+(f_P-f_{PL})\sqrt{\frac{([P]_t+[L]_t+K_d)^2}{4}-[P]_t[L]_t}$$

Here, F means the normalized fluorescence intensity and $[L]_t$ the total ligand concentration in the particular titration step. $[P]_t$ as mutein concentration, f_{PL} as fluorescence coefficient of the mutein-ligand complex and K_d as the thermodynamic dissociation constant of said complex were fitted as free parameters to the normalized data.

Fig. 1 represents graphically the results of the fluorescence titrations of the DigA16 mutein with the ligands digoxigenin, digitoxigenin and ouabain. It turns out that digitoxigenin is bound even tighter than digoxigenin, while no binding is observed for ouabain.

The values resulting from the fluorescence titrations for the dissociation constants of the complexes of the bilin-binding protein muteins and the various ligands are summarized in the following table:

Bbp variant	Ligand	K_d [nM]
Bbp:	digoxigenin	-*
DigA:	digoxigenin	295 ± 37
	digoxin	200 ± 34
DigA16:	digoxigenin	30.2 ± 3.6
	digoxin	31.1 ± 3.2
	digitoxigenin	2.8 ± 2.7
	digitoxin	2.7 ± 2.0
	ouabain	-*

testosterone	-*
4-aminofluroescein	-*

* no detectable binding activity

Example 4

Preparation of fusion proteins of the DigA16 mutein and bacterial alkaline phosphatase and use for detecting digoxigenin groups in an ELISA and in a Western blot

In order to produce two different fusion proteins of the DigA16 mutein and bacterial alkaline phosphatase (PhoA) with different arrangement of the partners within the polypeptide chain, the two expression plasmids pBBP27 and pBBP29 were constructed by using the molecular-biological methods familiar to the skilled worker.

pBB27 codes for a fusion protein of PhoA including the signal sequence thereof, a short peptide linker having the amino acid sequence Pro-Pro-Ser-ala, the sequence corresponding to the mature DigA16 mutein and the Strep tag II. A relevant section of the pBBP27 nucleic acid sequence is represented, together with the encoded amino acid sequence, as SEQ ID NO: 17 in the sequence listing. The section begins with the *Xba*I cleavage site and ends with the *Hind*III cleavage site. The vector elements outside this region are identical to vector pBBP21.

pBB29 codes for a fusion protein of DigA16 with preceding OmpA signal sequence, followed by the peptide sequence for Strep tag II, a sequence of 5 glycine residues and the mature PhoA sequence without the N-terminal amino acid arginine. A relevant section of the pBBP29 nucleic acid sequence is represented, together with the encoded amino acid sequence, as SEQ ID NO: 18 in the sequence listing. The section begins with the

*Xba*I cleavage site and ends with the *Hind*III cleavage site. The vector elements outside this region are identical to vector pBBP21.

Both plasmids additionally code for the bacterial protein disulfide isomerase DsbC on a separate cistron located in 3′ direction. The plasmids are shown diagrammatically in **Fig.** 2.

The fusion proteins encoded by plasmids pBBP27 and pBBP29 were produced analogously to the method for preparing the simple muteins, described in example 3. In order to avoid complexing the metal ions from the active center of PhoA, lysis of the bacterial periplasm was carried out using EDTA-free lysis buffer. Polymyxin B sulfate (2 mg/ml, Sigma) was added to the buffer as an agent destabilizing the outer cell membrane. All other buffers used for purification were likewise EDTA-free.

The fusion proteins purified by affinity chromatography by means of the Strep tag II were dialyzed against PBS buffer overnight. The fusion protein yields were between 100 and 200 µg per 2 l of culture medium. The purity of the fusion proteins obtained was checked by SDS polyacrylamide gel electrophoresis, according to example 3, and determined at 90-95%. Subsequently, the fusion proteins were used for directly detecting conjugates of the digoxigenin group with various proteins both in a sandwich ELISA and in a Western blot.

While the conjugates used of digoxigenin with RNaseA and BSA were prepared according to example 1, a conjugate of digoxigenin with ovalbumin (Sigma) was prepared by adding 1.5 µmol (0.99 mg) DIG-NHS in 25 µl of DMSO in µl steps and with constant mixing to 300 nmol (13.5 mg) of ovalbumin in 1.9 ml of 5% sodium hydrogen carbonate. The mixture was incubated with

stirring at RT for 1 h. Excess reagent was removed from the ovalbumin conjugate via a PD-10 gel filtration column according to the manufacturer's instructions.

For detecting digoxigenin groups in a sandwich ELISA, the wells in in each case two vertical columns of a microtiter plate (ELISA strips, 2×8 well with high binding capacity, F type, Greiner) were filled in each case with 100 µl of a 100 µg/ml solution of the BSA-digoxigenin conjugate or the ovalbumin-digoxigenin conjugate in PBS and incubated at RT overnight. As a control, the wells of a fifth vertical row of the microtiter plate were filled with 100 µl of a 100 µg/ml solution of nonconjugated BSA (Sigma) in PBS and likewise incubated at RT overnight. After removing the solution, unoccupied binding sites were saturated with 200 µl of a solution of 2% w/v BSA in PBST for 2 h. After washing three times with PBST, 50 µl of a 1 µM solution of the purified fusion protein were in each case introduced into the first well of a row, and the Tween concentration was adjusted to 0.1% v/v by adding 1 µl of a solution of 5% v/v Tween. The subsequent wells in each row were initially charged with 50 µl of PBST. Then, 50 µl of the purified fusion protein were pipetted in each case into the second well, mixed and, starting therefrom, 1:2 dilutions were prepared stepwise in the other wells of the vertical row. After incubation at RT for 1 h, the wells were washed twice with PBST and twice with PBS. The fusion proteins bound to the digoxigenin groups were finally detected by means of alkaline phosphatase-catalyzed hydrolysis of p-nitrophenyl phosphate. For this purpose, 100 µl of a solution of 0.5 mg/ml p-nitrophenyl phosphate (Amresco) in AP buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris/HCl pH 8.8) were introduced into the wells and product formation was monitored by measuring absorption at 405 nm in a SpectraMax 250 photometer (Molecular Devices).

Fig. 3 shows the result of this measurement. According to this, the digoxigenin group is recognized both as conjugate with BSA and as conjugate with ovalbumin, leading to the conclusion that binding by the DigA16 mutein is context-independent. Furthermore, both fusion proteins are active both with regard to the binding function for the digoxigenin group and enzymatically and produce, despite their different structure, almost identical signals.

The fusion proteins encoded by vectors pBBP27 and pBBP29 were used in a Western blot by first fractionating 5 µl of a protein mixture in PBS, whose concentration of digoxigenin-BSA conjugate, digoxigenin-ovalbumin conjugate and digoxigenin-RNaseA conjugate was simultaneously in each case 100 µg/ml, and also 5 µl of a protein mixture in PBS, whose concentration of underivatized BSA, ovalbumin and RNaseA likewise was simultaneously in each case 100 µg/ml, by SDS polyacrylamide gel electrophoresis. The protein mixture was then transferred to nitrocellulose by electrotransfer (Blake et al., Anal. Biochem. 136 (1984), 175-179). The membrane was then washed in 10 ml of PBST for three times 5 min and incubated with 10 ml of a 0.5 µM solution of in each case one of the two fusion proteins for 1 h. The membrane was then washed in 10 ml PBST for two times 5 min and in 10 ml of PBS for two times 5 min and finally gently agitated in 10 ml of AP buffer for 10 min. For the chromogenic detection reaction, the membrane was incubated in 10 ml of AP buffer to which 30 µl BCIP (50 µg/ml in dimethylformamide) and 5 µl NBT (75 µg/ml in 70% v/v dimethylformamide) had been added, and in this way bound fusion protein was detected.

Fig. 4 shows the result of this detection method. It turns out again that binding of the digoxigenin group by the two fusion proteins is independent of the carrier protein and that both fusion proteins achieve

comparable signal intensities. The same carrier proteins cause no signal whatsoever if they are not conjugated with the digoxigenin group.

SEQUENCE LISTING

GENERAL INFORMATION:

APPLICANT:
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TELEFAX: 08161-714352

TITLE OF THE INVENTION: Muteins of the bilin-binding protein

NUMBER OF SEQUENCES: 18

COMPUTER READABLE FORM:
MEDIUM TYPE: Floppy disk
COMPUTER: IBM PC compatible
OPERATING SYSTEM: PC-DOS/MS-DOS
SOFTWARE: Microsoft Word, format: Text

CURRENT APPLICATION DATA:
APPLICATION NUMBER: not yet known
FILING DATE: not yet known

INFORMATION FOR SEQ ID NO:1:

SEQUENCE CHARACTERISTICS:
LENGTH: 1219 base pairs
TYPE: nucleic acid
STRANDEDNESS: double
TOPOLOGY: linear
MOLECULE TYPE: fragment of phasmid pBBP20

FEATURE:
NAME/KEY: signal peptide
LOCATION: (22..84)

FEATURE:
NAME/KEY: mature peptide
LOCATION: (85..1209)
OTHER INFORMATION:
/Product = "fusion protein composed of bilin-binding protein Strep-tag II and fragment of phage envelope protein pIII"
/Codon = (sequence: "TAG", amino acid:Gln)

FEATURE:
NAME/KEY: coding sequence
LOCATION: (85..606)
OTHER INFORMATION:
/Product = "mature bilin-binding protein"

FEATURE:
NAME/KEY: coding sequence
LOCATION: (607..636)
OTHER INFORMATION:
/Product = "Strep-tag II affinity tag"

FEATURE:
NAME/KEY: coding sequence
LOCATION: (637..639)
OTHER INFORMATION:
/Other = "amber stop codon"

FEATURE:
NAME/KEY: coding sequence
LOCATION: (640..1209)
OTHER INFORMATION:
/Product = "amino acids 217-406 of envelope protein pIII"

SEQUENCE DESCRIPTION: SEQ ID NO:1:

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          5                  10                  15

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         20                  25                  30

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Pro Asn Ser Val Glu Lys Tyr Gly Lys Cys Gly Trp Ala Glu Tyr
         35                  40                  45

ACT CCT GAA GGC AAG AGT GTC AAA GTT TCG AAC TAC CAC GTA ATC  270
Thr Pro Glu Gly Lys Ser Val Lys Val Ser Asn Tyr His Val Ile
         50                  55                  60

CAC GGC AAG GAA TAC TTT ATT GAA GGA ACT GCC TAC CCA GTT GGT  315
His Gly Lys Glu Tyr Phe Ile Glu Gly Thr Ala Tyr Pro Val Gly
         65                  70                  75

GAC TCC AAG ATT GGA AAG ATC TAC CAC AGC CTG ACT TAC GGA GGT  360
Asp Ser Lys Ile Gly Lys Ile Tyr His Ser Leu Thr Tyr Gly Gly
         80                  85                  90

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Val Thr Lys Glu Asn Val Phe Asn Val Leu Ser Thr Asp Asn Lys
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Thr Gly Glu Ala Lys Thr Ala Val Glu Asn Tyr Leu Ile Gly Ser
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        185                 190                 195

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        200                 205                 210

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Ser Glu Gly Gly Gly Ser Gly Gly Gly Ser Gly Ser Gly Asp Phe
        215                 220                 225

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Asp Tyr Glu Lys Met Ala Asn Ala Asn Lys Gly Ala Met Thr Glu
        230                 235                 240

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Asn Ala Asp Glu Asn Ala Leu Gln Ser Asp Ala Lys Gly Lys Leu
        245                 250                 255

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Asp Ser Val Ala Thr Asp Tyr Gly Ala Ala Ile Asp Gly Phe Ile
        260                 265                 270

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        275                 280                 285

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Phe Ala Gly Ser Asn Ser Gln Met Ala Gln Val Gly Asp Gly Asp
        290                 295                 300

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Asn Ser Pro Leu Met Asn Asn Phe Arg Gln Tyr Leu Pro Ser Leu
        305                 310                 315
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CCA TAT GAA TTT TCT ATT GAT TGT GAC AAA ATA AAC TTA TTC CGT 1125
Pro Tyr Glu Phe Ser Ile Asp Cys Asp Lys Ile Asn Leu Phe Arg
        335                 340                 345

GGT GTC TTT GCG TTT CTT TTA TAT GTT GCC ACC TTT ATG TAT GTA 1170
Gly Val Phe Ala Phe Leu Leu Tyr Val Ala Thr Phe Met Tyr Val
        350                 355                 360

TTT TCT ACG TTT GCT AAC ATA CTG CGT AAT AAG GAG TCT         1209
Phe Ser Thr Phe Ala Asn Ile Leu Arg Asn Lys Glu Ser
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INFORMATION FOR SEQ ID NO:2:

SEQUENCE CHARACTERISTICS:
LENGTH: 64 bases
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: synthetic oligodeoxynucleotide

SEQUENCE DESCRIPTION: SEQ ID NO:2

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INFORMATION FOR SEQ ID NO:3:

SEQUENCE CHARACTERISTICS:
LENGTH: 71 bases
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: synthetic oligodeoxynucleotide

SEQUENCE DESCRIPTION: SEQ ID NO:3:

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INFORMATION FOR SEQ ID NO:4:

SEQUENCE CHARACTERISTICS:
LENGTH: 74 bases
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: synthetic oligodeoxynucleotide

SEQUENCE DESCRIPTION: SEQ ID NO:4:

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INFORMATION FOR SEQ ID NO:5:

SEQUENCE CHARACTERISTICS:
LENGTH: 78 bases
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: synthetic oligodeoxynucleotide

SEQUENCE DESCRIPTION: SEQ ID NO:5:

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INFORMATION FOR SEQ ID NO:6:

SEQUENCE CHARACTERISTICS:
LENGTH: 36 bases
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: synthetic oligodeoxynucleotide

SEQUENCE DESCRIPTION: SEQ ID NO:6:

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INFORMATION FOR SEQ ID NO:7:

SEQUENCE CHARACTERISTICS:
LENGTH: 37 bases
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: synthetic oligodeoxynucleotide

SEQUENCE DESCRIPTION: SEQ ID NO:7:

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INFORMATION FOR SEQ ID NO:8:

SEQUENCE CHARACTERISTICS:
LENGTH: 46 bases
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: synthetic oligodeoxynucleotide

SEQUENCE DESCRIPTION: SEQ ID NO:8:

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INFORMATION FOR SEQ ID NO:9:

SEQUENCE CHARACTERISTICS:
LENGTH: 793 base pairs
TYPE: nucleic acid
STRANDEDNESS: double
TOPOLOGY: linear
MOLECULE TYPE: fragment of plasmid pBBP22

FEATURE:
NAME/KEY: signal peptide
LOCATION: (22..84)

FEATURE:
NAME/KEY: mature peptide
LOCATION: (85..783)
OTHER INFORMATION:
/Product = "fusion protein composed of bilin-binding protein, Strep-tag II and albumin-binding domain"

FEATURE:
NAME/KEY: coding sequence
LOCATION: (85..606)
OTHER INFORMATION:
/Product = "mature bilin-binding protein"

FEATURE:
NAME/KEY: coding sequence
LOCATION: (607..636)
OTHER INFORMATION:
/Product = "Strep-tag II affinity tag"

FEATURE:
NAME/KEY: coding sequence
LOCATION: (637..783)
OTHER INFORMATION:
/Product = "albumin-binding domain of protein G"

SEQUENCE DESCRIPTION: SEQ ID NO:9:

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            -10                 -5              -1   1

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Tyr His Asp Gly Ala Cys Pro Glu Val Lys Pro Val Asp Asn Phe
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Thr Pro Glu Gly Lys Ser Val Lys Val Ser Asn Tyr His Val Ile
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CAC GGC AAG GAA TAC TTT ATT GAA GGA ACT GCC TAC CCA GTT GGT 315
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GAC TCC AAG ATT GGA AAG ATC TAC CAC AGC CTG ACT TAC GGA GGT 360
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GGA CAC CAA GAC TTC GTC TGG GTG CTC TCC AGA AGC ATG GTC CTT 495
Gly His Gln Asp Phe Val Trp Val Leu Ser Arg Ser Met Val Leu
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CCA GTA GTC GAC TCC CAG AAA CTG GTA TAC AGT GAC TTC TCT GAA 585
Pro Val Val Asp Ser Gln Lys Leu Val Tyr Ser Asp Phe Ser Glu
155 160 165

GCC GCC TGC AAG GTC AAC AAT AGC AAC TGG TCT CAC CCG CAG TTC 630
Ala Ala Cys Lys Val Asn Asn Ser Asn Trp Ser His Pro Gln Phe
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Glu Lys Pro Ala Ser Leu Ala Glu Ala Lys Val Leu Ala Asn Arg
185 190 195

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Glu Leu Asp Lys Tyr Gly Val Ser Asp Tyr Tyr Lys Asn Leu Ile
200 205 210

AAC AAC GCT AAA ACC GTT GAA GGT GTT AAA GCT CTG ATC GAC GAA 765
Asn Asn Ala Lys Thr Val Glu Gly Val Lys Ala Leu Ile Asp Glu
215 220 225

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Ile Leu Ala Ala Leu Pro
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INFORMATION FOR SEQ ID NO:10:

SEQUENCE CHARACTERISTICS:
LENGTH: 17 bases
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: synthetic oligodeoxynucleotide

SEQUENCE DESCRIPTION: SEQ ID NO:10:

GACGGTGCCT GTCCCGA 17

INFORMATION FOR SEQ ID NO:11:

SEQUENCE CHARACTERISTICS:
LENGTH: 17 bases
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: synthetic oligodeoxynucleotide

SEQUENCE DESCRIPTION: SEQ ID NO:11:

GACTACTGGG GAGCCGA 17

INFORMATION FOR SEQ ID NO:12:

SEQUENCE CHARACTERISTICS:
LENGTH: 522
TYPE: nucleic acid
STRANDEDNESS: double
TOPOLOGY: linear
MOLECULE TYPE: coding sequence of mutein DigA

FEATURE:
NAME/KEY: coding sequence
LOCATION: (1..522)
OTHER INFORMATION:
/Product = "mutein without fusion parts"

SEQUENCE DESCRIPTION: SEQ ID NO:12:

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  1               5                  10                  15

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Asn Phe Asp Trp Ser Gln Tyr His Gly Lys Trp Trp Glu Val Ala
                 20                  25                  30
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                 35                  40                  45

GAG TAC ACT CCT GAA GGC AAG AGT GTC AAA GTT TCG CGC TAC TCT 180
Glu Tyr Thr Pro Glu Gly Lys Ser Val Lys Val Ser Arg Tyr Ser
                 50                  55                  60

GTA ATC CAC GGC AAG GAA TAC TTT TCC GAA GGT ACC GCC TAC CCA 225
Val Ile His Gly Lys Glu Tyr Phe Ser Glu Gly Thr Ala Tyr Pro
                 65                  70                  75

GTT GGT GAC TCC AAG ATT GGA AAG ATC TAC CAC AGC TAC ACT ATT 270
Val Gly Asp Ser Lys Ile Gly Lys Ile Tyr His Ser Tyr Thr Ile
                 80                  85                  90

GGA GGT GTG ACC CAG GAG GGT GTA TTC AAC GTA CTC TCC ACT GAC 315
Gly Gly Val Thr Gln Glu Gly Val Phe Asn Val Leu Ser Thr Asp
                 95                 100                 105

AAC AAG AAC TAC ATC ATC GGA TAC TTT TGC TCG TAC GAC GAG GAC 360
Asn Lys Asn Tyr Ile Ile Gly Tyr Phe Cys Ser Tyr Asp Glu Asp
                110                 115                 120

AAG AAG GGA CAC ATG GAC TTG GTC TGG GTG CTC TCC AGA AGC ATG 405
Lys Lys Gly His Met Asp Leu Val Trp Val Leu Ser Arg Ser Met
                125                 130                 135

GTC CTT ACT GGT GAA GCC AAG ACC GCT GTC GAG AAC TAC CTT ATC 450
Val Leu Thr Gly Glu Ala Lys Thr Ala Val Glu Asn Tyr Leu Ile
                140                 145                 150

GGC TCC CCA GTA GTC GAC TCC CAG AAA CTG GTA TAC AGT GAC TTC 495
Gly Ser Pro Val Val Asp Ser Gln Lys Leu Val Tyr Ser Asp Phe
                155                 160                 165

TCT GAA GCC GCC TGC AAG GTC AAC AAT                         522
Ser Glu Ala Ala Cys Lys Val Asn Asn
                170
```

INFORMATION FOR SEQ ID NO:13:

SEQUENCE CHARACTERISTICS:
LENGTH: 76 bases
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: synthetic oligodeoxynucleotide

SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
CTGGTCCCAG TACCATGGTA AATGGTGGNN KGTCGCCNNK TACCCCNNKN    50
NKNNKNNKAA GTACGGAAAG TGCGGA                              76
```

INFORMATION FOR SEQ ID NO:14:

SEQUENCE CHARACTERISTICS:
LENGTH: 1219 base pairs
TYPE: nucleic acid
STRANDEDNESS: double
TOPOLOGY: linear
MOLECULE TYPE: fragment of phasmid pBBP24

FEATURE:
NAME/KEY: signal peptide
LOCATION: (22..84)

FEATURE:
NAME/KEY: mature peptide
LOCATION: (85..1209)
OTHER INFORMATION:
/Product = "fusion protein composed of bilin-binding protein Strep-tag II and fragment of phage envelope protein pIII, with interrupted reading frame"
/Codon = (sequence: "TAG", amino acid:Gln)

FEATURE:
NAME/KEY: coding sequence
LOCATION: (85..606)
OTHER INFORMATION:
/Product = "mature bilin-binding protein with interrupted reading frame"

FEATURE:
NAME/KEY: coding sequence
LOCATION: (607..636)
OTHER INFORMATION:
/Product = "Strep-tag II affinity tag"

FEATURE:
NAME/KEY: coding sequence
LOCATION: (637..639)
OTHER INFORMATION:
/Product = "amber stop codon

FEATURE:
NAME/KEY: coding sequence
LOCATION: (640..1209)
OTHER INFORMATION:
/Product = "amino acids 217-406 of envelope protein pIII"

EQUENCE DESCRIPTION: SEQ ID NO:14:

```
TCTAGATAAC GAGGGCAAAA A ATG AAA AAG ACA GCT ATC GCG ATT       45
                        Met Lys Lys Thr Ala Ile Ala Ile
                        -21 -20                 -15

GCA GTG GCA CTG GCT GGT TTC GCT ACC GTA GCG CAG GCC GAC GTG   90
Ala Val Ala Leu Ala Gly Phe Ala Thr Val Ala Gln Ala Asp Val
            -10                 -5              -1   1
```

```
TAC CAC GAC GGT GCC TGT CCC GAA GTC AAG CCA GTC GAC AAC TTC 135
Tyr His Asp Gly Ala Cys Pro Glu Val Lys Pro Val Asp Asn Phe
         5                  10                  15

GAC TGG TCC CAG TAC CAT GGT AAA TGG TGG GAA GTC GCC AAA TAC 180
Asp Trp Ser Gln Tyr His Gly Lys Trp Trp Glu Val Ala Lys Tyr
        20                  25                  30

CCC AAC TCA GTT GAG AAG TAC GGA AAT TAA TGA TGG GCT GAG TAC 225
Pro Asn Ser Val Glu Lys Tyr Gly Asn         Trp Ala Glu Tyr
        35                  40                  45

ACT CCT GAA GGC AAG AGT GTC AAA GTT TCG AAC TAC CAC GTA ATC 270
Thr Pro Glu Gly Lys Ser Val Lys Val Ser Asn Tyr His Val Ile
        50                  55                  60

CAC GGC AAG GAA TAC TTT ATT GAA GGA ACT GCC TAC CCA GTT GGT 315
His Gly Lys Glu Tyr Phe Ile Glu Gly Thr Ala Tyr Pro Val Gly
        65                  70                  75

GAC TCC AAG ATT GGA AAG ATC TAC CAC AGC CTG ACT TAC GGA GGT 360
Asp Ser Lys Ile Gly Lys Ile Tyr His Ser Leu Thr Tyr Gly Gly
        80                  85                  90

GTC ACC AAG GAG AAC GTA TTC AAC GTA CTC TCC ACT GAC AAC AAG 405
Val Thr Lys Glu Asn Val Phe Asn Val Leu Ser Thr Asp Asn Lys
        95                 100                 105

AAC TAC ATC ATC GGA TAC TAC TGC AAA TAC GAC GAG GAC AAG AAG 450
Asn Tyr Ile Ile Gly Tyr Tyr Cys Lys Tyr Asp Glu Asp Lys Lys
       110                 115                 120

GGA CAC CAA GAC TTC GTC TGG GTG CTC TCC AGA AGC ATG GTC CTT 495
Gly His Gln Asp Phe Val Trp Val Leu Ser Arg Ser Met Val Leu
       125                 130                 135

ACT GGT GAA GCC AAG ACC GCT GTC GAG AAC TAC CTT ATC GGC TCC 540
Thr Gly Glu Ala Lys Thr Ala Val Glu Asn Tyr Leu Ile Gly Ser
       140                 145                 150

CCA GTA GTC GAC TCC CAG AAA CTG GTA TAC AGT GAC TTC TCT GAA 585
Pro Val Val Asp Ser Gln Lys Leu Val Tyr Ser Asp Phe Ser Glu
       155                 160                 165

GCC GCC TGC AAG GTC AAC AAT AGC AAC TGG TCT CAC CCG CAG TTC 630
Ala Ala Cys Lys Val Asn Asn Ser Asn Trp Ser His Pro Gln Phe
       170                 175                 180

GAA AAA TAG GCT GGC GGC GGC TCT GGT GGT GGT TCT GGC GGC GGC 675
Glu Lys Gln Ala Gly Gly Gly Ser Gly Gly Gly Ser Gly Gly Gly
       185                 190                 195

TCT GAG GGT GGT GGC TCT GAG GGT GGC GGT TCT GAG GGT GGC GGC 720
Ser Glu Gly Gly Gly Ser Glu Gly Gly Gly Ser Glu Gly Gly Gly
       200                 205                 210
```

TCT GAG GGA GGC GGT TCC GGT GGT GGC TCT GGT TCC GGT GAT TTT 765
Ser Glu Gly Gly Gly Ser Gly Gly Gly Ser Gly Ser Gly Asp Phe
215 220 225

GAT TAT GAA AAG ATG GCA AAC GCT AAT AAG GGG GCT ATG ACC GAA 810
Asp Tyr Glu Lys Met Ala Asn Ala Asn Lys Gly Ala Met Thr Glu
230 235 240

AAT GCC GAT GAA AAC GCG CTA CAG TCT GAC GCT AAA GGC AAA CTT 855
Asn Ala Asp Glu Asn Ala Leu Gln Ser Asp Ala Lys Gly Lys Leu
245 250 255

GAT TCT GTC GCT ACT GAT TAC GGT GCT GCT ATC GAT GGT TTC ATT 900
Asp Ser Val Ala Thr Asp Tyr Gly Ala Ala Ile Asp Gly Phe Ile
260 265 270

GGT GAC GTT TCC GGC CTT GCT AAT GGT AAT GGT GCT ACT GGT GAT 945
Gly Asp Val Ser Gly Leu Ala Asn Gly Asn Gly Ala Thr Gly Asp
275 280 285

TTT GCT GGC TCT AAT TCC CAA ATG GCT CAA GTC GGT GAC GGT GAT 990
Phe Ala Gly Ser Asn Ser Gln Met Ala Gln Val Gly Asp Gly Asp
290 295 300

AAT TCA CCT TTA ATG AAT AAT TTC CGT CAA TAT TTA CCT TCC CTC 1035
Asn Ser Pro Leu Met Asn Asn Phe Arg Gln Tyr Leu Pro Ser Leu
305 310 315

CCT CAA TCG GTT GAA TGT CGC CCT TTT GTC TTT GGC GCT GGT AAA 1080
Pro Gln Ser Val Glu Cys Arg Pro Phe Val Phe Gly Ala Gly Lys
320 325 330

CCA TAT GAA TTT TCT ATT GAT TGT GAC AAA ATA AAC TTA TTC CGT 1125
Pro Tyr Glu Phe Ser Ile Asp Cys Asp Lys Ile Asn Leu Phe Arg
335 340 345

GGT GTC TTT GCG TTT CTT TTA TAT GTT GCC ACC TTT ATG TAT GTA 1170
Gly Val Phe Ala Phe Leu Leu Tyr Val Ala Thr Phe Met Tyr Val
350 355 360

TTT TCT ACG TTT GCT AAC ATA CTG CGT AAT AAG GAG TCT 1209
Phe Ser Thr Phe Ala Asn Ile Leu Arg Asn Lys Glu Ser
365 370 375

TAATAAGCTT 1219

INFORMATION FOR SEQ ID NO:15:

SEQUENCE CHARACTERISTICS:
LENGTH: 522
TYPE: nucleic acid
STRANDEDNESS: double
TOPOLOGY: linear
MOLECULE TYPE: coding sequence of mutein DigA16

FEATURE:
NAME/KEY: coding sequence
LOCATION: (1..522)
OTHER INFORMATION:
/Product = "mutein without fusion parts"

SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
GAC GTG TAC CAC GAC GGT GCC TGT CCC GAA GTC AAG CCA GTC GAC  45
Asp Val Tyr His Asp Gly Ala Cys Pro Glu Val Lys Pro Val Asp
 1               5                  10                  15

AAC TTC GAC TGG TCC CAG TAC CAT GGT AAA TGG TGG CAG GTC GCC  90
Asn Phe Asp Trp Ser Gln Tyr His Gly Lys Trp Trp Gln Val Ala
                20                  25                  30

GCG TAC CCC GAT CAT ATT ACG AAG TAC GGA AAG TGC GGA TGG GCT 135
Ala Tyr Pro Asp His Ile Thr Lys Tyr Gly Lys Cys Gly Trp Ala
                35                  40                  45

GAG TAC ACT CCT GAA GGC AAG AGT GTC AAA GTT TCG CGC TAC TCT 180
Glu Tyr Thr Pro Glu Gly Lys Ser Val Lys Val Ser Arg Tyr Ser
                50                  55                  60

GTA ATC CAC GGC AAG GAA TAC TTT TCC GAA GGT ACC GCC TAC CCA 225
Val Ile His Gly Lys Glu Tyr Phe Ser Glu Gly Thr Ala Tyr Pro
                65                  70                  75

GTT GGT GAC TCC AAG ATT GGA AAG ATC TAC CAC AGC TAC ACT ATT 270
Val Gly Asp Ser Lys Ile Gly Lys Ile Tyr His Ser Tyr Thr Ile
                80                  85                  90

GGA GGT GTG ACC CAG GAG GGT GTA TTC AAC GTA CTC TCC ACT GAC 315
Gly Gly Val Thr Gln Glu Gly Val Phe Asn Val Leu Ser Thr Asp
                95                 100                 105

AAC AAG AAC TAC ATC ATC GGA TAC TTT TGC TCG TAC GAC GAG GAC 360
Asn Lys Asn Tyr Ile Ile Gly Tyr Phe Cys Ser Tyr Asp Glu Asp
               110                 115                 120

AAG AAG GGA CAC ATG GAC TTG GTC TGG GTG CTC TCC AGA AGC ATG 405
Lys Lys Gly His Met Asp Leu Val Trp Val Leu Ser Arg Ser Met
               125                 130                 135

GTC CTT ACT GGT GAA GCC AAG ACC GCT GTC GAG AAC TAC CTT ATC 450
Val Leu Thr Gly Glu Ala Lys Thr Ala Val Glu Asn Tyr Leu Ile
               140                 145                 150

GGC TCC CCA GTA GTC GAC TCC CAG AAA CTG GTA TAC AGT GAC TTC 495
Gly Ser Pro Val Val Asp Ser Gln Lys Leu Val Tyr Ser Asp Phe
               155                 160                 165

TCT GAA GCC GCC TGC AAG GTC AAC AAT                         522
Ser Glu Ala Ala Cys Lys Val Asn Asn
               170
```

INFORMATION FOR SEQ ID NO:16

SEQUENCE CHARACTERISTICS:
LENGTH: 1380 base pairs
TYPE: nucleic acid
STRANDEDNESS: double
TOPOLOGY: linear
MOLECULE TYPE: fragment of plasmid pBBP21

FEATURE:
NAME/KEY: signal peptide
LOCATION: (22..84)

FEATURE:
NAME/KEY: mature peptide
LOCATION: (85..636)
OTHER INFORMATION:
/Product = "fusion protein composed of bilin-binding protein Strep-tag II"

FEATURE:
NAME/KEY: signal peptide
LOCATION: (658..717)

FEATURE:
NAME/KEY: mature peptide
LOCATION: (718..1365)
OTHER INFORMATION:
/Product = "DsbC protein"

SEQUENCE DESCRIPTION: SEQ ID NO:16:

```
TCTAGATAAC GAGGGCAAAA A ATG AAA AAG ACA GCT ATC GCG ATT      45
                        Met Lys Lys Thr Ala Ile Ala Ile
                        -21 -20                 -15

GCA GTG GCA CTG GCT GGT TTC GCT ACC GTA GCG CAG GCC GAC GTG  90
Ala Val Ala Leu Ala Gly Phe Ala Thr Val Ala Gln Ala Asp Val
            -10                 -5                  -1   1

TAC CAC GAC GGT GCC TGT CCC GAA GTC AAG CCA GTC GAC AAC TTC 135
Tyr His Asp Gly Ala Cys Pro Glu Val Lys Pro Val Asp Asn Phe
          5                  10                  15

GAC TGG TCC CAG TAC CAT GGT AAA TGG TGG GAA GTC GCC AAA TAC 180
Asp Trp Ser Gln Tyr His Gly Lys Trp Trp Glu Val Ala Lys Tyr
         20                  25                  30

CCC AAC TCA GTT GAG AAG TAC GGA AAG TGC GGA TGG GCT GAG TAC 225
Pro Asn Ser Val Glu Lys Tyr Gly Lys Cys Gly Trp Ala Glu Tyr
         35                  40                  45

ACT CCT GAA GGC AAG AGT GTC AAA GTT TCG AAC TAC CAC GTA ATC 270
Thr Pro Glu Gly Lys Ser Val Lys Val Ser Asn Tyr His Val Ile
         50                  55                  60
```

```
CAC GGC AAG GAA TAC TTT ATT GAA GGA ACT GCC TAC CCA GTT GGT 315
His Gly Lys Glu Tyr Phe Ile Glu Gly Thr Ala Tyr Pro Val Gly
         65                  70                  75

GAC TCC AAG ATT GGA AAG ATC TAC CAC AGC CTG ACT TAC GGA GGT 360
Asp Ser Lys Ile Gly Lys Ile Tyr His Ser Leu Thr Tyr Gly Gly
         80                  85                  90

GTC ACC AAG GAG AAC GTA TTC AAC GTA CTC TCC ACT GAC AAC AAG 405
Val Thr Lys Glu Asn Val Phe Asn Val Leu Ser Thr Asp Asn Lys
         95                 100                 105

AAC TAC ATC ATC GGA TAC TAC TGC AAA TAC GAC GAG GAC AAG AAG 450
Asn Tyr Ile Ile Gly Tyr Tyr Cys Lys Tyr Asp Glu Asp Lys Lys
        110                 115                 120

GGA CAC CAA GAC TTC GTC TGG GTG CTC TCC AGA AGC ATG GTC CTT 495
Gly His Gln Asp Phe Val Trp Val Leu Ser Arg Ser Met Val Leu
        125                 130                 135

ACT GGT GAA GCC AAG ACC GCT GTC GAG AAC TAC CTT ATC GGC TCC 540
Thr Gly Glu Ala Lys Thr Ala Val Glu Asn Tyr Leu Ile Gly Ser
        140                 145                 150

CCA GTA GTC GAC TCC CAG AAA CTG GTA TAC AGT GAC TTC TCT GAA 585
Pro Val Val Asp Ser Gln Lys Leu Val Tyr Ser Asp Phe Ser Glu
        155                 160                 165

GCC GCC TGC AAG GTC AAC AAT AGC AAC TGG TCT CAC CCG CAG TTC 630
Ala Ala Cys Lys Val Asn Asn Ser Asn Trp Ser His Pro Gln Phe
        170                 175                 180

GAA AAA TAATAAGCTT CGGGAAGATT T ATG AAG AAA GGT TTT ATG 675
Glu Lys                         Met Lys Lys Gly Phe Met
                                -20                 -15

TTG TTT ACT TTG TTA GCG GCG TTT TCA GGC TTT GCT CAG GCT GAT 720
Leu Phe Thr Leu Leu Ala Ala Phe Ser Gly Phe Ala Gln Ala Asp
                -10                  -5                  -1  1

GAC GCG GCA ATT CAA CAA ACG TTA GCC AAA ATG GGC ATC AAA AGC 765
Asp Ala Ala Ile Gln Gln Thr Leu Ala Lys Met Gly Ile Lys Ser
             5                  10                  15

AGC GAT ATT CAG CCC GCG CCT GTA GCT GGC ATG AAG ACA GTT CTG 810
Ser Asp Ile Gln Pro Ala Pro Val Ala Gly Met Lys Thr Val Leu
            20                  25                  30

ACT AAC AGC GGC GTG TTG TAC ATC ACC GAT GAT GGT AAA CAT ATC 855
Thr Asn Ser Gly Val Leu Tyr Ile Thr Asp Asp Gly Lys His Ile
            35                  40                  45

ATT CAG GGG CCA ATG TAT GAC GTT AGT GGC ACG GCT CCG GTC AAT 900
Ile Gln Gly Pro Met Tyr Asp Val Ser Gly Thr Ala Pro Val Asn
            50                  55                  60
```

```
GTC ACC AAT AAG ATG CTG TTA AAG CAG TTG AAT GCG CTT GAA AAA 945
Val Thr Asn Lys Met Leu Leu Lys Gln Leu Asn Ala Leu Glu Lys
             65                  70                  75

GAG ATG ATC GTT TAT AAA GCG CCG CAG GAA AAA CAC GTC ATC ACC 990
Glu Met Ile Val Tyr Lys Ala Pro Gln Glu Lys His Val Ile Thr
             80                  85                  90

GTG TTT ACT GAT ATT ACC TGT GGT TAC TGC CAC AAA CTG CAT GAG 1035
Val Phe Thr Asp Ile Thr Cys Gly Tyr Cys His Lys Leu His Glu
             95                 100                 105

CAA ATG GCA GAC TAC AAC GCG CTG GGG ATC ACC GTG CGT TAT CTT 1080
Gln Met Ala Asp Tyr Asn Ala Leu Gly Ile Thr Val Arg Tyr Leu
            110                 115                 120

GCT TTC CCG CGC CAG GGG CTG GAC AGC GAT GCA GAG AAA GAA ATG 1125
Ala Phe Pro Arg Gln Gly Leu Asp Ser Asp Ala Glu Lys Glu Met
            125                 130                 135

AAA GCT ATC TGG TGT GCG AAA GAT AAA AAC AAA GCG TTT GAT GAT 1170
Lys Ala Ile Trp Cys Ala Lys Asp Lys Asn Lys Ala Phe Asp Asp
            140                 145                 150

GTG ATG GCA GGT AAA AGC GTC GCA CCA GCC AGT TGC GAC GTG GAT 1215
Val Met Ala Gly Lys Ser Val Ala Pro Ala Ser Cys Asp Val Asp
            155                 160                 165

ATT GCC GAC CAT TAC GCA CTT GGC GTC CAG CTT GGC GTT AGC GGT 1260
Ile Ala Asp His Tyr Ala Leu Gly Val Gln Leu Gly Val Ser Gly
            170                 175                 180

ACT CCG GCA GTT GTG CTG AGC AAT GGC ACA CTT GTT CCG GGT TAC 1305
Thr Pro Ala Val Val Leu Ser Asn Gly Thr Leu Val Pro Gly Tyr
            185                 190                 195

CAG CCG CCG AAA GAG ATG AAA GAA TTC CTC GAC GAA CAC CAA AAA 1350
Gln Pro Pro Lys Glu Met Lys Glu Phe Leu Asp Glu His Gln Lys
            200                 205                 210

ATG ACC AGC GGT AAA TAATTCGCGT AGCTT                        1380
Met Thr Ser Gly Lys
            215
```

INFORMATION FOR SEQ ID NO:17:

SEQUENCE CHARACTERISTICS:
LENGTH: 2009 base pairs
TYPE: nucleic acid
STRANDEDNESS: double
TOPOLOGY: linear
MOLECULE TYPE: fragment of plasmid pBBP27

FEATURE:
NAME/KEY: signal peptide
LOCATION: (23..85)

FEATURE:
NAME/KEY: mature peptide
LOCATION: (86..1999)
OTHER INFORMATION:
/Product = "fusion protein composed of alkaline phosphatase, linker peptide Pro-Pro-Ser-Ala, mutein DigA16 and Strep tag II"

FEATURE:
NAME/KEY: coding sequence
LOCATION: (86..1435)
OTHER INFORMATION:
/Product = "linker peptide Pro-Pro-Ser-Ala"

FEATURE:
NAME/KEY: coding sequence
LOCATION: (1448..1969)
OTHER INFORMATION:
/Product = "DigA16 mutein"

FEATURE:
NAME/KEY: coding sequence
LOCATION: (1970..1999)
OTHER INFORMATION:
/Product = "Strep-tag II affinity tag"

SEQUENCE DESCRIPTION: SEQ ID NO:17:

```
TCTAGAACAT GGAGAAAATA AA GTG AAA CAA AGC ACT ATT GCA CTG      46
                         Val Lys Gln Ser Thr Ile Ala Leu
                         -21 -20                 -15

GCA CTC TTA CCG TTA CTG TTT ACC CCT GTG ACA AAA GCC CGG ACA   91
Ala Leu Leu Pro Leu Leu Phe Thr Pro Val Thr Lys Ala Arg Thr
            -10                 -5              -1   1

CCA GAA ATG CCT GTT CTG GAA AAC CGG GCT GCT CAG GGC GAT ATT  136
Pro Glu Met Pro Val Leu Glu Asn Arg Ala Ala Gln Gly Asp Ile
          5                  10                  15

ACT GCA CCC GGC GGT GCT CGC CGT TTA ACG GGT GAT CAG ACT GCC  181
Thr Ala Pro Gly Gly Ala Arg Arg Leu Thr Gly Asp Gln Thr Ala
         20                  25                  30

GCT CTG CGT GAT TCT CTT AGC GAT AAA CCT GCA AAA AAT ATT ATT  226
Ala Leu Arg Asp Ser Leu Ser Asp Lys Pro Ala Lys Asn Ile Ile
         35                  40                  45

TTG CTG ATT GGC GAT GGG ATG GGG GAC TCG GAA ATT ACT GCC GCA  271
Leu Leu Ile Gly Asp Gly Met Gly Asp Ser Glu Ile Thr Ala Ala
         50                  55                  60
```

```
CGT AAT TAT GCC GAA GGT GCG GGC GGC TTT TTT AAA GGT ATA GAT 316
Arg Asn Tyr Ala Glu Gly Ala Gly Gly Phe Phe Lys Gly Ile Asp
         65                  70                  75

GCC TTA CCG CTT ACC GGG CAA TAC ACT CAC TAT GCG CTG AAT AAA 361
Ala Leu Pro Leu Thr Gly Gln Tyr Thr His Tyr Ala Leu Asn Lys
         80                  85                  90

AAA ACC GGC AAA CCG GAC TAC GTC ACC GAC TCG GCT GCA TCA GCA 406
Lys Thr Gly Lys Pro Asp Tyr Val Thr Asp Ser Ala Ala Ser Ala
         95                 100                 105

ACC GCC TGG TCA ACC GGT GTC AAA ACC TAT AAC GGC GCG CTG GGC 451
Thr Ala Trp Ser Thr Gly Val Lys Thr Tyr Asn Gly Ala Leu Gly
        110                 115                 120

GTC GAT ATT CAC GAA AAA GAT CAC CCA ACG ATT CTG GAA ATG GCA 496
Val Asp Ile His Glu Lys Asp His Pro Thr Ile Leu Glu Met Ala
        125                 130                 135

AAA GCC GCA GGT CTG GCG ACC GGT AAC GTT TCT ACC GCA GAG TTG 541
Lys Ala Ala Gly Leu Ala Thr Gly Asn Val Ser Thr Ala Glu Leu
        140                 145                 150

CAG GAT GCC ACG CCC GCT GCG CTG GTG GCA CAT GTG ACC TCG CGC 586
Gln Asp Ala Thr Pro Ala Ala Leu Val Ala His Val Thr Ser Arg
        155                 160                 165

AAA TGC TAC GGT CCG AGC GCG ACC AGT GAA AAA TGT CCG GGT AAC 631
Lys Cys Tyr Gly Pro Ser Ala Thr Ser Glu Lys Cys Pro Gly Asn
        170                 175                 180

GCT CTG GAA AAA GGC GGA AAA GGA TCG ATT ACC GAA CAG CTG CTT 676
Ala Leu Glu Lys Gly Gly Lys Gly Ser Ile Thr Glu Gln Leu Leu
        185                 190                 195

AAC GCT CGT GCC GAC GTT ACG CTT GGC GGC GGC GCA AAA ACC TTT 721
Asn Ala Arg Ala Asp Val Thr Leu Gly Gly Gly Ala Lys Thr Phe
        200                 205                 210

GCT GAA ACG GCA ACC GCT GGT GAA TGG CAG GGA AAA ACG CTG CGT 766
Ala Glu Thr Ala Thr Ala Gly Glu Trp Gln Gly Lys Thr Leu Arg
        215                 220                 225

GAA CAG GCA CAG GCG CGT GGT TAT CAG TTG GTG AGC GAT GCT GCC 811
Glu Gln Ala Gln Ala Arg Gly Tyr Gln Leu Val Ser Asp Ala Ala
        230                 235                 240

TCA CTG AAT TCG GTG ACG GAA GCG AAT CAG CAA AAA CCC CTG CTT 856
Ser Leu Asn Ser Val Thr Glu Ala Asn Gln Gln Lys Pro Leu Leu
        245                 250                 255

GGC CTG TTT GCT GAC GGC AAT ATG CCA GTG CGC TGG CTA GGA CCG 901
Gly Leu Phe Ala Asp Gly Asn Met Pro Val Arg Trp Leu Gly Pro
        260                 265                 270
```

```
AAA GCA ACG TAC CAT GGC AAT ATC GAT AAG CCC GCA GTC ACC TGT 946
Lys Ala Thr Tyr His Gly Asn Ile Asp Lys Pro Ala Val Thr Cys
        275                 280                 285

ACG CCA AAT CCG CAA CGT AAT GAC AGT GTA CCA ACC CTG GCG CAG 991
Thr Pro Asn Pro Gln Arg Asn Asp Ser Val Pro Thr Leu Ala Gln
        290                 295                 300

ATG ACC GAC AAA GCC ATT GAA TTG TTG AGT AAA AAT GAG AAA GGC 1036
Met Thr Asp Lys Ala Ile Glu Leu Leu Ser Lys Asn Glu Lys Gly
        305                 310                 315

TTT TTC CTG CAA GTT GAA GGT GCG TCA ATC GAT AAA CAG GAT CAT 1081
Phe Phe Leu Gln Val Glu Gly Ala Ser Ile Asp Lys Gln Asp His
        320                 325                 330

GCT GCG AAT CCT TGT GGG CAA ATT GGC GAG ACG GTC GAT CTC GAT 1126
Ala Ala Asn Pro Cys Gly Gln Ile Gly Glu Thr Val Asp Leu Asp
        335                 340                 345

GAA GCC GTA CAA CGG GCG CTG GAA TTC GCT AAA AAG GAG GGT AAC 1171
Glu Ala Val Gln Arg Ala Leu Glu Phe Ala Lys Lys Glu Gly Asn
        350                 355                 360

ACG CTG GTC ATA GTC ACC GCT GAT CAC GCC CAC GCC AGC CAG ATT 1216
Thr Leu Val Ile Val Thr Ala Asp His Ala His Ala Ser Gln Ile
        365                 370                 375

GTT GCG CCG GAT ACC AAA GCT CCG GGC CTC ACC CAG GCG CTA AAT 1261
Val Ala Pro Asp Thr Lys Ala Pro Gly Leu Thr Gln Ala Leu Asn
        380                 385                 390

ACC AAA GAT GGC GCA GTG ATG GTG ATG AGT TAC GGG AAC TCC GAA 1306
Thr Lys Asp Gly Ala Val Met Val Met Ser Tyr Gly Asn Ser Glu
        395                 400                 405

GAG GAT TCA CAA GAA CAT ACC GGC AGT CAG TTG CGT ATT GCG GCG 1351
Glu Asp Ser Gln Glu His Thr Gly Ser Gln Leu Arg Ile Ala Ala
        410                 415                 420

TAT GGC CCG CAT GCC GCC AAT GTT GTT GGA CTG ACC GAC CAG ACC 1396
Tyr Gly Pro His Ala Ala Asn Val Val Gly Leu Thr Asp Gln Thr
        425                 430                 435

GAT CTC TTC TAC ACC ATG AAA GCC GCT CTG GGG CTG AAA CCG CCT 1441
Asp Leu Phe Tyr Thr Met Lys Ala Ala Leu Gly Leu Lys Pro Pro
        440                 445                 450

AGC GCT GAC GTG TAC CAC GAC GGT GCC TGT CCC GAA GTC AAG CCA 1486
Ser Ala Asp Val Tyr His Asp Gly Ala Cys Pro Glu Val Lys Pro
        455                 460                 465

GTC GAC AAC TTC GAC TGG TCC CAG TAC CAT GGT AAA TGG TGG CAG 1531
Val Asp Asn Phe Asp Trp Ser Gln Tyr His Gly Lys Trp Trp Gln
        470                 475                 480
```

```
GTC GCC GCG TAC CCC GAT CAT ATT ACG AAG TAC GGA AAG TGC GGA 1576
Val Ala Ala Tyr Pro Asp His Ile Thr Lys Tyr Gly Lys Cys Gly
        485                 490                 495

TGG GCT GAG TAC ACT CCT GAA GGC AAG AGT GTC AAA GTT TCG CGC 1621
Trp Ala Glu Tyr Thr Pro Glu Gly Lys Ser Val Lys Val Ser Arg
        500                 505                 510

TAC TCT GTA ATC CAC GGC AAG GAA TAC TTT TCC GAA GGT ACC GCC 1666
Tyr Ser Val Ile His Gly Lys Glu Tyr Phe Ser Glu Gly Thr Ala
        515                 520                 525

TAC CCA GTT GGT GAC TCC AAG ATT GGA AAG ATC TAC CAC AGC TAC 1711
Tyr Pro Val Gly Asp Ser Lys Ile Gly Lys Ile Tyr His Ser Tyr
        530                 535                 540

ACT ATT GGA GGT GTG ACC CAG GAG GGT GTA TTC AAC GTA CTC TCC 1756
Thr Ile Gly Gly Val Thr Gln Glu Gly Val Phe Asn Val Leu Ser
        545                 550                 555

ACT GAC AAC AAG AAC TAC ATC ATC GGA TAC TTT TGC TCG TAC GAC 1801
Thr Asp Asn Lys Asn Tyr Ile Ile Gly Tyr Phe Cys Ser Tyr Asp
        560                 565                 570

GAG GAC AAG AAG GGA CAC ATG GAC TTG GTC TGG GTG CTC TCC AGA 1846
Glu Asp Lys Lys Gly His Met Asp Leu Val Trp Val Leu Ser Arg
        575                 580                 585

AGC ATG GTC CTT ACT GGT GAA GCC AAG ACC GCT GTC GAG AAC TAC 1891
Ser Met Val Leu Thr Gly Glu Ala Lys Thr Ala Val Glu Asn Tyr
        590                 595                 600

CTT ATC GGC TCC CCA GTA GTC GAC TCC CAG AAA CTG GTA TAC AGT 1936
Leu Ile Gly Ser Pro Val Val Asp Ser Gln Lys Leu Val Tyr Ser
        605                 610                 615

GAC TTC TCT GAA GCC GCC TGC AAG GTC AAC AAT AGC AAC TGG TCT 1981
Asp Phe Ser Glu Ala Ala Cys Lys Val Asn Asn Ser Asn Trp Ser
        620                 625                 630

CAC CCG CAG TTC GAA AAA TAATAAGCTT                          2009
His Pro Gln Phe Glu Lys
        635
```

INFORMATION FOR SEQ ID NO:18:

SEQUENCE CHARACTERISTICS:
LENGTH: 2005 base pairs
TYPE: nucleic acid
STRANDEDNESS: double
TOPOLOGY: linear
MOLECULE TYPE: fragment of plasmid pBBP29

FEATURE:
NAME/KEY: signal peptide
LOCATION: (22..84)

```
FEATURE:
   NAME/KEY: mature peptide
   LOCATION: (85..1998)
   OTHER INFORMATION:
   /Product = "fusion protein composed of DigA16 mutein, Strep
               tag II, linker peptide Gly(5) and alkaline
               phosphatase"

FEATURE:
   NAME/KEY: coding sequence
   LOCATION: (85..606)
   OTHER INFORMATION:
   /Product = "DigA16 mutein"

FEATURE:
   NAME/KEY: coding sequence
   LOCATION: (607..636)
   OTHER INFORMATION:
   /Product = "Strep-tag II affinity tag"

FEATURE:
   NAME/KEY: coding sequence
   LOCATION: (637..651)
   OTHER INFORMATION:
   /Product = "linker peptide Gly-Gly-Gly-Gly-Gly"

FEATURE:
   NAME/KEY: coding sequence
   LOCATION: (652..1998)
   OTHER INFORMATION:
   /Product = "alkaline phosphatase without signal sequence and
               N-terminal Arg"

SEQUENCE DESCRIPTION: SEQ ID NO:18:

TCTAGATAAC GAGGGCAAAA A ATG AAA AAG ACA GCT ATC GCG ATT      45
                        Met Lys Lys Thr Ala Ile Ala Ile
                        -21 -20                 -15

GCA GTG GCA CTG GCT GGT TTC GCT ACC GTA GCG CAG GCC GAC GTG  90
Ala Val Ala Leu Ala Gly Phe Ala Thr Val Ala Gln Ala Asp Val
            -10                  -5              -1   1

TAC CAC GAC GGT GCC TGT CCC GAA GTC AAG CCA GTC GAC AAC TTC 135
Tyr His Asp Gly Ala Cys Pro Glu Val Lys Pro Val Asp Asn Phe
          5                  10                  15

GAC TGG TCC CAG TAC CAT GGT AAA TGG TGG CAG GTC GCC GCG TAC 180
Asp Trp Ser Gln Tyr His Gly Lys Trp Trp Gln Val Ala Ala Tyr
         20                  25                  30

CCC GAT CAT ATT ACG AAG TAC GGA AAG TGC GGA TGG GCT GAG TAC 225
Pro Asp His Ile Thr Lys Tyr Gly Lys Cys Gly Trp Ala Glu Tyr
         35                  40                  45

ACT CCT GAA GGC AAG AGT GTC AAA GTT TCG CGC TAC TCT GTA ATC 270
Thr Pro Glu Gly Lys Ser Val Lys Val Ser Arg Tyr Ser Val Ile
         50                  55                  60
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CAC GGC AAG GAA TAC TTT TCC GAA GGT ACC GCC TAC CCA GTT GGT 315
His Gly Lys Glu Tyr Phe Ser Glu Gly Thr Ala Tyr Pro Val Gly
         65                  70                  75

GAC TCC AAG ATT GGA AAG ATC TAC CAC AGC TAC ACT ATT GGA GGT 360
Asp Ser Lys Ile Gly Lys Ile Tyr His Ser Tyr Thr Ile Gly Gly
         80                  85                  90

GTG ACC CAG GAG GGT GTA TTC AAC GTA CTC TCC ACT GAC AAC AAG 405
Val Thr Gln Glu Gly Val Phe Asn Val Leu Ser Thr Asp Asn Lys
         95                 100                 105

AAC TAC ATC ATC GGA TAC TTT TGC TCG TAC GAC GAG GAC AAG AAG 450
Asn Tyr Ile Ile Gly Tyr Phe Cys Ser Tyr Asp Glu Asp Lys Lys
        110                 115                 120

GGA CAC ATG GAC TTG GTC TGG GTG CTC TCC AGA AGC ATG GTC CTT 495
Gly His Met Asp Leu Val Trp Val Leu Ser Arg Ser Met Val Leu
        125                 130                 135

ACT GGT GAA GCC AAG ACC GCT GTC GAG AAC TAC CTT ATC GGC TCC 540
Thr Gly Glu Ala Lys Thr Ala Val Glu Asn Tyr Leu Ile Gly Ser
        140                 145                 150

CCA GTA GTC GAC TCC CAG AAA CTG GTA TAC AGT GAC TTC TCT GAA 585
Pro Val Val Asp Ser Gln Lys Leu Val Tyr Ser Asp Phe Ser Glu
        155                 160                 165

GCC GCC TGC AAG GTC AAC AAT AGC AAC TGG TCT CAC CCG CAG TTC 630
Ala Ala Cys Lys Val Asn Asn Ser Asn Trp Ser His Pro Gln Phe
        170                 175                 180

GAA AAA GGT GGC GGC GGT GGT ACA CCA GAA ATG CCT GTT CTG GAA 675
Glu Lys Gly Gly Gly Gly Gly Thr Pro Glu Met Pro Val Leu Glu
        185                 190                 195

AAC CGG GCT GCT CAG GGC GAT ATT ACT GCA CCC GGC GGT GCT CGC 720
Asn Arg Ala Ala Gln Gly Asp Ile Thr Ala Pro Gly Gly Ala Arg
        200                 205                 210

CGT TTA ACG GGT GAT CAG ACT GCC GCT CTG CGT GAT TCT CTT AGC 765
Arg Leu Thr Gly Asp Gln Thr Ala Ala Leu Arg Asp Ser Leu Ser
        215                 220                 225

GAT AAA CCT GCA AAA AAT ATT ATT TTG CTG ATT GGC GAT GGG ATG 810
Asp Lys Pro Ala Lys Asn Ile Ile Leu Leu Ile Gly Asp Gly Met
        230                 235                 240

GGG GAC TCG GAA ATT ACT GCC GCA CGT AAT TAT GCC GAA GGT GCG 855
Gly Asp Ser Glu Ile Thr Ala Ala Arg Asn Tyr Ala Glu Gly Ala
        245                 250                 255

GGC GGC TTT TTT AAA GGT ATA GAT GCC TTA CCG CTT ACC GGG CAA 900
Gly Gly Phe Phe Lys Gly Ile Asp Ala Leu Pro Leu Thr Gly Gln
        260                 265                 270
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TAC ACT CAC TAT GCG CTG AAT AAA AAA ACC GGC AAA CCG GAC TAC 945
Tyr Thr His Tyr Ala Leu Asn Lys Lys Thr Gly Lys Pro Asp Tyr
        275                 280                 285

GTC ACC GAC TCG GCT GCA TCA GCA ACC GCC TGG TCA ACC GGT GTC 990
Val Thr Asp Ser Ala Ala Ser Ala Thr Ala Trp Ser Thr Gly Val
        290                 295                 300

AAA ACC TAT AAC GGC GCG CTG GGC GTC GAT ATT CAC GAA AAA GAT 1035
Lys Thr Tyr Asn Gly Ala Leu Gly Val Asp Ile His Glu Lys Asp
        305                 310                 315

CAC CCA ACG ATT CTG GAA ATG GCA AAA GCC GCA GGT CTG GCG ACC 1080
His Pro Thr Ile Leu Glu Met Ala Lys Ala Ala Gly Leu Ala Thr
        320                 325                 330

GGT AAC GTT TCT ACC GCA GAG TTG CAG GAT GCC ACG CCC GCT GCG 1125
Gly Asn Val Ser Thr Ala Glu Leu Gln Asp Ala Thr Pro Ala Ala
        335                 340                 345

CTG GTG GCA CAT GTG ACC TCG CGC AAA TGC TAC GGT CCG AGC GCG 1170
Leu Val Ala His Val Thr Ser Arg Lys Cys Tyr Gly Pro Ser Ala
        350                 355                 360

ACC AGT GAA AAA TGT CCG GGT AAC GCT CTG GAA AAA GGC GGA AAA 1215
Thr Ser Glu Lys Cys Pro Gly Asn Ala Leu Glu Lys Gly Gly Lys
        365                 370                 375

GGA TCG ATT ACC GAA CAG CTG CTT AAC GCT CGT GCC GAC GTT ACG 1260
Gly Ser Ile Thr Glu Gln Leu Leu Asn Ala Arg Ala Asp Val Thr
        380                 385                 390

CTT GGC GGC GGC GCA AAA ACC TTT GCT GAA ACG GCA ACC GCT GGT 1305
Leu Gly Gly Gly Ala Lys Thr Phe Ala Glu Thr Ala Thr Ala Gly
        395                 400                 405

GAA TGG CAG GGA AAA ACG CTG CGT GAA CAG GCA CAG GCG CGT GGT 1350
Glu Trp Gln Gly Lys Thr Leu Arg Glu Gln Ala Gln Ala Arg Gly
        410                 415                 420

TAT CAG TTG GTG AGC GAT GCT GCC TCA CTG AAT TCG GTG ACG GAA 1395
Tyr Gln Leu Val Ser Asp Ala Ala Ser Leu Asn Ser Val Thr Glu
        425                 430                 435

GCG AAT CAG CAA AAA CCC CTG CTT GGC CTG TTT GCT GAC GGC AAT 1440
Ala Asn Gln Gln Lys Pro Leu Leu Gly Leu Phe Ala Asp Gly Asn
        440                 445                 450

ATG CCA GTG CGC TGG CTA GGA CCG AAA GCA ACG TAC CAT GGC AAT 1485
Met Pro Val Arg Trp Leu Gly Pro Lys Ala Thr Tyr His Gly Asn
        455                 460                 465

ATC GAT AAG CCC GCA GTC ACC TGT ACG CCA AAT CCG CAA CGT AAT 1530
Ile Asp Lys Pro Ala Val Thr Cys Thr Pro Asn Pro Gln Arg Asn
        470                 475                 480
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GAC AGT GTA CCA ACC CTG GCG CAG ATG ACC GAC AAA GCC ATT GAA 1575
Asp Ser Val Pro Thr Leu Ala Gln Met Thr Asp Lys Ala Ile Glu
        485                 490                 495

TTG TTG AGT AAA AAT GAG AAA GGC TTT TTC CTG CAA GTT GAA GGT 1620
Leu Leu Ser Lys Asn Glu Lys Gly Phe Phe Leu Gln Val Glu Gly
        500                 505                 510

GCG TCA ATC GAT AAA CAG GAT CAT GCT GCG AAT CCT TGT GGG CAA 1665
Ala Ser Ile Asp Lys Gln Asp His Ala Ala Asn Pro Cys Gly Gln
        515                 520                 525

ATT GGC GAG ACG GTC GAT CTC GAT GAA GCC GTA CAA CGG GCG CTG 1710
Ile Gly Glu Thr Val Asp Leu Asp Glu Ala Val Gln Arg Ala Leu
        530                 535                 540

GAA TTC GCT AAA AAG GAG GGT AAC ACG CTG GTC ATA GTC ACC GCT 1755
Glu Phe Ala Lys Lys Glu Gly Asn Thr Leu Val Ile Val Thr Ala
        545                 550                 555

GAT CAC GCC CAC GCC AGC CAG ATT GTT GCG CCG GAT ACC AAA GCT 1800
Asp His Ala His Ala Ser Gln Ile Val Ala Pro Asp Thr Lys Ala
        560                 565                 570

CCG GGC CTC ACC CAG GCG CTA AAT ACC AAA GAT GGC GCA GTG ATG 1845
Pro Gly Leu Thr Gln Ala Leu Asn Thr Lys Asp Gly Ala Val Met
        575                 580                 585

GTG ATG AGT TAC GGG AAC TCC GAA GAG GAT TCA CAA GAA CAT ACC 1890
Val Met Ser Tyr Gly Asn Ser Glu Glu Asp Ser Gln Glu His Thr
        590                 595                 600

GGC AGT CAG TTG CGT ATT GCG GCG TAT GGC CCG CAT GCC GCC AAT 1935
Gly Ser Gln Leu Arg Ile Ala Ala Tyr Gly Pro His Ala Ala Asn
        605                 610                 615

GTT GTT GGA CTG ACC GAC CAG ACC GAT CTC TTC TAC ACC ATG AAA 1980
Val Val Gly Leu Thr Asp Gln Thr Asp Leu Phe Tyr Thr Met Lys
        620                 625                 630

GCC GCT CTG GGG CTG AAA TAAGCTT                             2005
Ala Ala Leu Gly Leu Lys
        635
```

Patent Claims

1. A polypeptide selected from muteins of the bilin-binding protein, characterized in that it

(a) is able to bind digoxigenin or digoxigenin conjugates,

(b) does not bind ouabain, testosterone and 4-aminofluorescein and

(c) has an amino acid substitution at at least one of the sequence positions 28, 31, 34, 35, 36, 37, 58, 60, 69, 88, 90, 95, 97, 114, 116, 125 and 127 of the bilin-binding protein.

2. The polypeptide as claimed in claim 1, characterized in that the dissociation constant of the complex with digoxigenin is 100 nM or less.

3. The polypeptide as claimed in claim 1 or 2, characterized in that it carries, when compared to the bilin-binding protein, at least one of the amino acid substitutions selected from Glu(28)->Gln, Lys(31)->Ala, Asn(34)->Asp, Ser(35)->His, Val(36)->Ile, Glu(37)->Thr, Asn(58)->Arg, His(60)->Ser, Ile(69)->Ser, Leu(88)->Tyr, Tyr(90)->Ile, Lys(95)->Gln, Asn(97)->Gly, Tyr(114)->Phe, Lys(116)->Ser, Gln(125)->Met and Phe(127)->Leu.

4. The polypeptide as claimed in one or more of claims 1 to 3, characterized in that it carries at least one label group, selected from enzymatic label, radioactive label, fluorescent label, chromophoric label, (bio)luminescent label or label containing haptens, biotin, metal complexes, metals or colloidal gold.

5. A fusion protein of polypeptides as claimed in one or more of claims 1 to 4, characterized in that an enzyme, another protein or a protein domain, a signal

sequence and/or an affinity peptide is fused to the amino terminus of the polypeptide in an operable manner.

6. A fusion protein of polypeptides as claimed in one or more of claims 1 to 5, characterized in that an enzyme, another protein or a protein domain, a targeting sequence and/or an affinity peptide is fused to the carboxy terminus of the polypeptide in an operable manner.

7. A nucleic acid, characterized in that it comprises a sequence coding for a mutein or a fusion protein of a mutein of the bilin-binding protein as claimed in one or more of claims 1 to 6.

8. A method for preparing a mutein or a fusion protein of a mutein of the bilin-binding protein as claimed in one or more of claims 1 to 6, characterized in that the nucleic acid coding for the mutein or the fusion protein of a mutein of the bilin-binding protein is expressed in a bacterial or eukaryotic host cell and the polypeptide is obtained from the cell or the culture supernatant.

9. The use of a mutein or a fusion protein of a mutein of the bilin-binding protein as claimed in one or more of claims 1 to 8 for binding, detecting, determining, immobilizing or removing digoxigenin or conjugates of digoxigenin with proteins, nucleic acids, carbohydrates, other biological or synthetic macromolecules or low molecular weight chemical compounds.

10. A method for detecting the digoxigenin group, wherein a mutein of the bilin-binding protein or a fusion protein of a mutein of the bilin-binding protein as claimed in one or more of claims 1 to 8 is contacted with digoxigenin or

with conjugates of digoxigenin under conditions suitable for effecting binding of the mutein to the digoxigenin group, and the mutein or the fusion protein of the mutein is determined.

4 pages(s) of drawings attached

Number: DE 199 26 068 C1
Int. Cl.^{7}: C 07 K 14/435
Published on: January 11, 2001

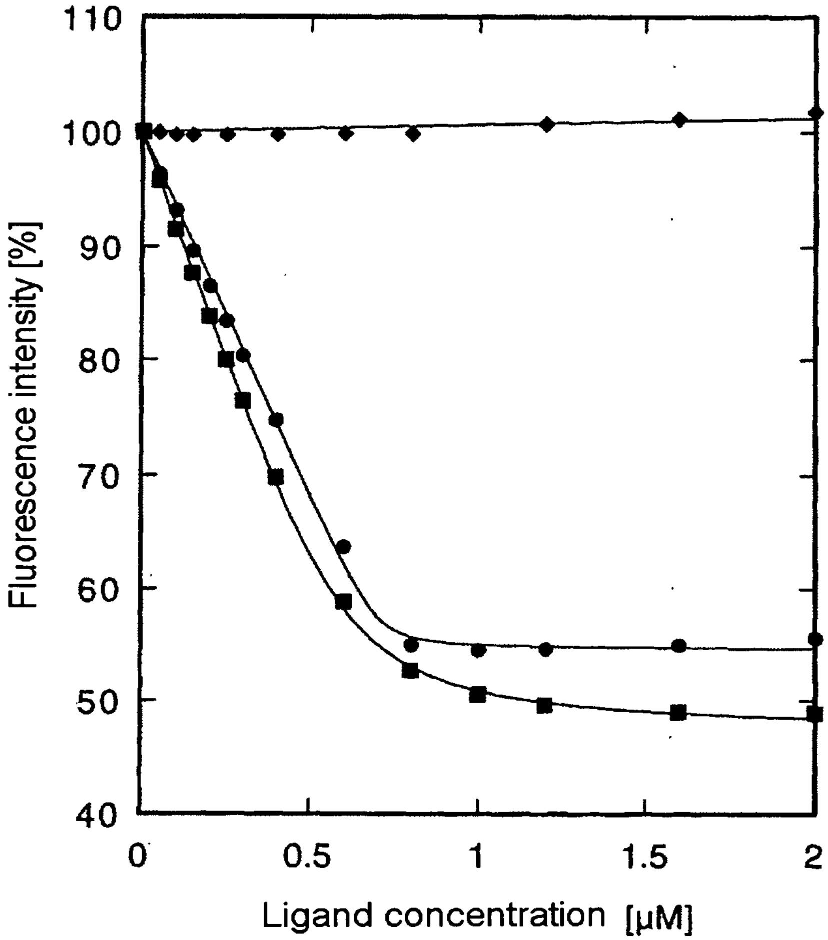

Figure 1

Number: DE 199 26 068 C1
Int. Cl.^{7}: C 07 K 14/435
Published on: January 11, 2001

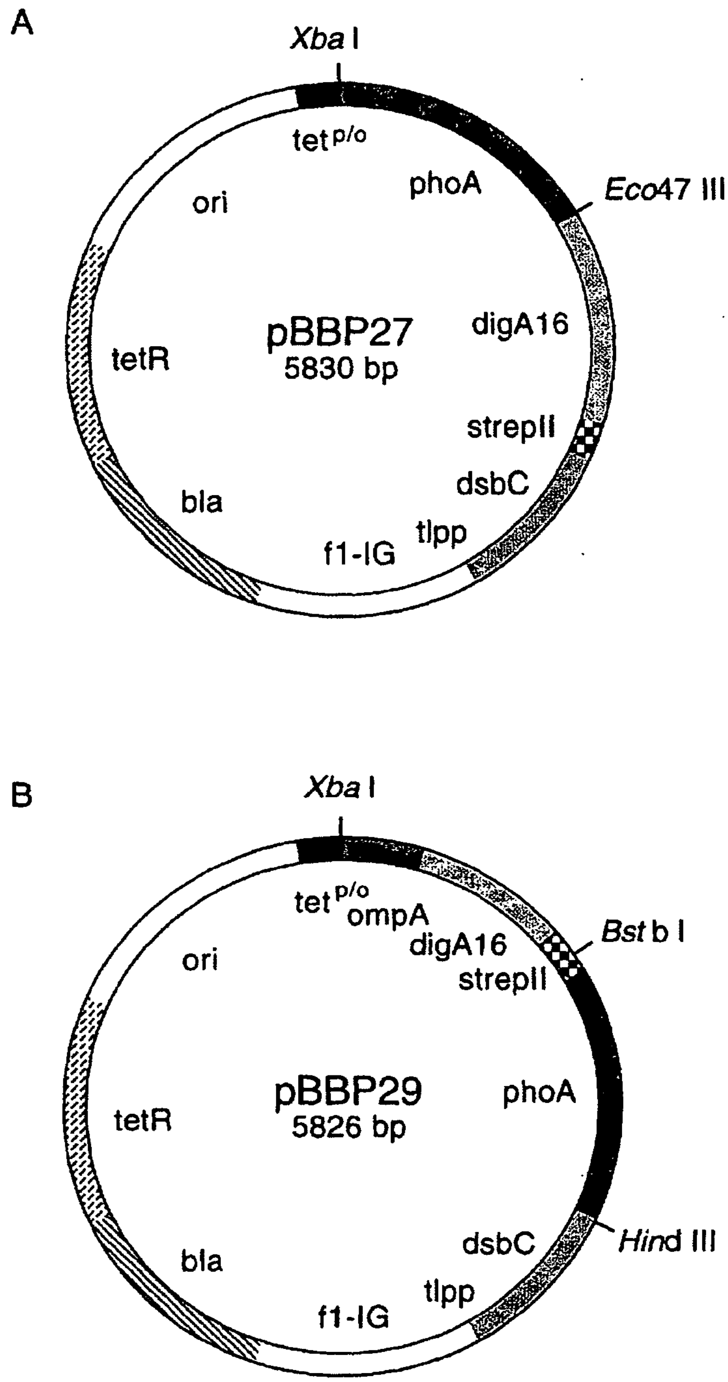

Figure 2

Number: DE 199 26 068 C1
Int. Cl.[7]: C 07 K 14/435
Published on: January 11, 2001

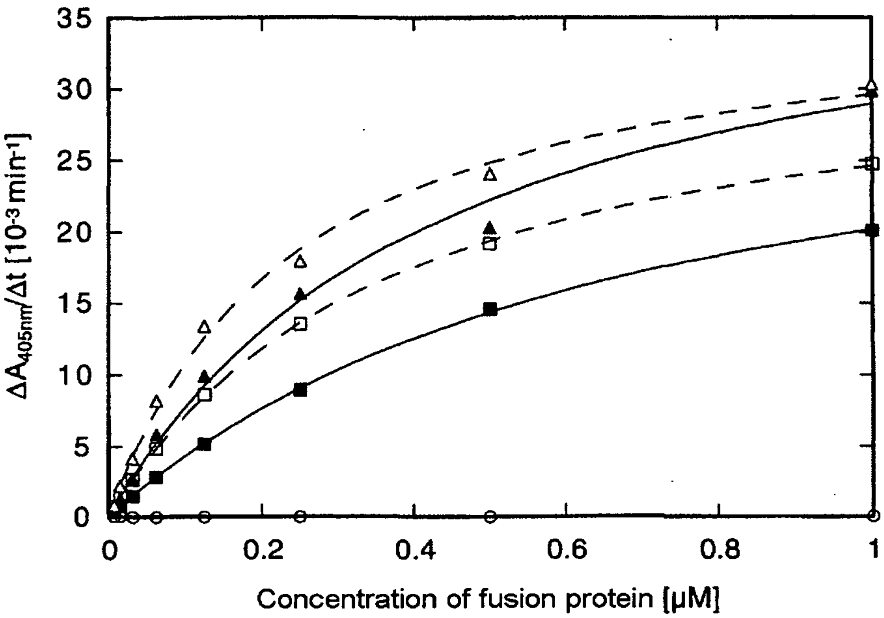

Figure 3

Number: DE 199 26 068 C1
Int. Cl.[7]: C 07 K 14/435
Published on: January 11, 2001

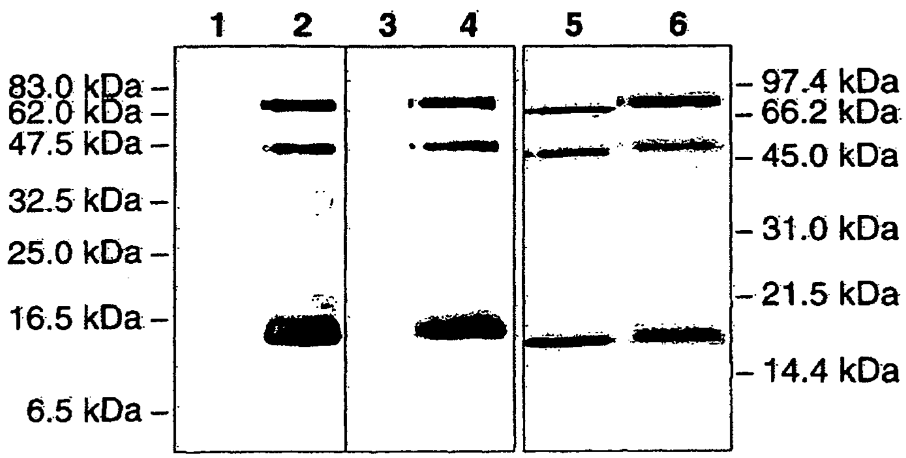

Figure 4